UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO

FACULTAD DE QUIMICA

"MECANISMOS DE LA NEUROTOXICIDAD DEL ROJO DE RUTENIO"

T E S I S

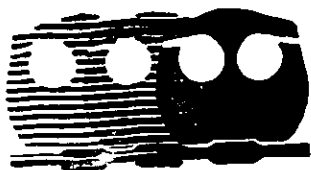QUE PARA OBTENER EL GRADO DE:

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PAGINACIÓN DISCONTINUA

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DOCTORADO EN BIOQUÍMICA

MECANISMOS DE LA NEUROTOXICIDAD DEL ROJO DE RUTENIO

JAIME IVÁN VELASCO VELÁZQUEZ

MÉXICO, D.F., JULIO DE 1998

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PRÓLOGO

El principal objetivo del presente trabajo fue establecer las características y los mecanismos de daño neuronal inducido por la exposición de neuronas en cultivo al colorante catiónico rojo de rutenio (RR). En la **Introducción** se describe la importancia de la muerte neuronal y los procesos que conducen a la degeneración celular, especialmente por muerte celular programada en el nemátodo *C. elegans* y por necrosis debida a excitotoxicidad. Asimismo, se describen los efectos del RR sobre sistemas biológicos, con especial énfasis en las acciones de este compuesto sobre el sistema nervioso (primer artículo de esta tesis). En la parte experimental se incluyen cuatro trabajos, dos publicados, uno sometido y otro en preparación. Para hacer referencia a ellos, decidí numerarlos (ver índice). El lector deberá consultar los trabajos que conforman la sección de **Resultados** para conocer la metodología, los hallazgos y la discusión de cada artículo. Para evitar ser repetitivo, en la **Discusión general** se trata de integrar todos los resultados y se proponen algunos sitios donde el RR podría actuar para producir la neurodegeneración. A lo largo de la tesis se usan abreviaturas, las cuales se definen en la página iii y, en general, también la primera vez que son mencionadas en el texto.

ÍNDICE

ABREVIATURAS

aa	aminoácido(s)
AMPA	ácido α-amino-3-hidroxi-5-metil-4-isoxazol propiónico
Ca^{2+}	ion calcio
$[Ca^{2+}]_i$	concentración intracelular de calcio
cDNA	DNA complementario
cols.	colaboradores
CPP32	caspasa 3
ER	retículo endoplásmico
Glu	glutamato
IC_{50}	concentración inhibitoria al 50%
ICE	enzima convertidora de interleucina 1B, caspasa 1
IP_3	inositol trifosfato
kb	kilobases
kDa	kilodaltones
La^{3+}	ion lantano
LCIC	liberación de calcio inducida por calcio
mRNA	RNA mensajero
MTT	bromuro de (3-(4,5-dimetiltiazol-2-il)-2,5-difenil tetrazolio
NMDA	N-metil-D-aspartato
NO	óxido nítrico
NOS	sintasa del óxido nítrico
$\cdot O_2^-$	radical superóxido
$\cdot OH$	radical hidroxilo
ONOOH	peroxinitrito
PARP	polimerasa de poli-ADP-ribosa
pb	pares de bases
ROS	especies reactivas de oxígeno
RR	rojo de rutenio
RyR	receptor a ryanodina
SN	sistema nervioso
SNC	sistema nervioso central
SR	retículo sarcoplásmico
t	tiempo
V	voltaje
VSCC	canales de calcio sensibles a voltaje

RESUMEN

El colorante inorgánico rojo de rutenio (RR) es un compuesto cargado positivamente que es capaz de disminuir la entrada de calcio (Ca^{2+}) en diversas preparaciones biológicas. El RR produce hiperexcitación y muerte neuronal al ser inyectado en distintas regiones cerebrales. En esta tesis se describen los efectos del RR sobre la supervivencia de neuronas y células gliales en cultivos primarios. El RR produjo muerte celular dependiente de la concentración (20-100 µM), en incubaciones de 8-24 horas en los cultivos neuronales, caracterizada por la desaparición de neuritas debida a la fragmentación de las mismas, daño de los somas neuronales, pérdida de la inmunorreactividad a una proteína que forma parte del citoesqueleto (α-tubulina) y unión del colorante al núcleo de las células afectadas. Encontramos que las neuronas corticales y granulares del cerebelo son igualmente susceptibles al RR, aunque en ambos tipos celulares siempre se observó una proporción de células resistentes a la acción tóxica del colorante. En contraste, los astrocitos cerebelosos en cultivo no fueron dañados por el RR. Esta toxicidad selectiva también se ha observado *in vivo*, en donde el RR dañó a las neuronas pero no a la glia presente en la zona inyectada. En cultivo, la entrada del colorante a los somas neuronales es un fenómeno que se observa aun cuando no se ha producido deterioro notable en la morfología. La hipótesis de que el RR se introduce a las neuronas previo a causar daño se ve apoyada por las siguientes observaciones: 1) los astrocitos en cultivo no internalizan el RR ni se mueren al estar en contacto con el colorante; 2) al prevenir la entrada del colorante a las neuronas con el trisialogangliósido GT1b, la neurodegeneración se previno parcialmente. Como posibles mecanismos para explicar la neurotoxicidad del RR, observamos que el colorante ocasiona distintas alteraciones en las neuronas corticales en cultivo, como son las alteraciones de la homeostasis del Ca^{2+} y una disminución de la actividad de la cadena de transporte de electrones, tanto en células como en mitocondrias aisladas de cerebro. No pudo establecerse una relación directa entre la acción inhibitoria del RR sobre canales de Ca^{2+}, ni de la participación de los receptores a glutamato, con la neurotoxicidad. La entrada del RR a las neuronas se estudió al expresar el RNA mensajero (mRNA) extraído de neuronas cultivadas en ovocitos de *Xenopus*, y exponer estas células a RR, ya sea en el medio o inyectándolo directamente dentro de los ovocitos. La inyección del mRNA neuronal en los ovocitos ocasionó la expresión de nuevas corrientes y un potencial de membrana más hiperpolarizado comparado con las células control. La exposición de los ovocitos a RR en el medio provocó una despolarización del potencial de membrana en ovocitos control e inyectados con mRNA, aunque en el último caso el cambio fue de mayor magnitud. Al introducir el colorante a los ovocitos mediante inyección, las células control no fueron afectadas por el colorante y las que expresaron el mRNA mostraron daño en aproximadamente un tercio de las células. Estos resultados sugieren que la entrada del RR a las neuronas no está mediada por proteínas, aunque una vez dentro de las células que expresan proteínas neuronales, el RR afecta de manera importante la supervivencia celular.

ABSTRACT

The inorganic dye ruthenium red (RR) is a highly charged positive compound able to block calcium (Ca^{2+}) entry on several biological systems. RR induces both hyperexcitation and neuronal death when administered into different brain regions. In this thesis, RR effects on cell survival in primary cultured neuronal and glial cells are described. RR caused a concentration-dependent (between 20 and 100 μM) neurodegeneration after 8-24 hours exposures to the dye. The neuronal death was characterized by fragmentation and loss of neurites, somata damage, remarkable diminution of the immunoreactivity to a cytoskeleton protein (α-tubulin) and RR binding to the nucleus of affected cells. Cortical and cerebellar granule neurons are equally sensitive to the damaging action of the dye. It is worth noting that in both cellular types, there is a proportion of neurons that is not affected by RR. In contrast, cerebellar astrocytes in culture are not damaged by RR. This selective toxicity is similar to that found *in vivo*, since RR damaged neurons but not astrocytes in the injected regions. In cultures, RR internalization to the somata of neurons is a phenomenon observed when no obvious alterations on morphology are seen. The hypothesis that RR entry to neurons is a determinant step in neurodegeneration is supported by the following observations: 1) cultured astrocytes do not internalize RR and are not affected by the dye. 2) when RR uptake by neurons is prevented by trisialoganglioside GT1b, the RR-induced neuronal death is diminished. The mechanisms through which RR might cause neurodegeneration are alterations of Ca^{2+} homeostasis, inhibition of electron chain function in cultured neurons and brain isolated mitochondrion. No direct correlation between the blocking action of RR on Ca^{2+} channels, or the activation of glutamate receptors, and neurotoxicity was observed. RR penetration to neurons was studied by injecting mRNA extracted from cultured neurons to *Xenopus* oocytes, and exposing those cells to RR extracellularly, or intracellularly by injection of the dye. Introduction of neuronal mRNA to oocytes caused the expression of novel currents and a more hyperpolarized membrane potential as compared with control cells. RR in the medium caused a depolarization of membrane potential in control and mRNA-injected oocytes, but in the latter case, the change was greater. When the dye was introduced to the oocytes, the control cells were not affected. In contrast, mRNA-expressing cells showed severe damage in about one third of the oocytes. Those results suggest that RR uptake by neurons is not mediated by a protein. However, once inside neuronal protein-expressing cells, RR is capable to induce severe cell damage.

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INTRODUCCIÓN

I. Muerte neuronal

La muerte de las neuronas es un fenómeno que se ha observado en asociación con la formación del Sistema Nervioso (SN), algunas patologías y el envejecimiento. En el caso del crecimiento, se ha descrito una muerte importante de neuronas (en algunos casos 50% o más de las células desaparecen) durante el correcto establecimiento de la morfología y de los circuitos neuronales. Es así que se ha propuesto que la muerte neuronal en el desarrollo del SN sirve para el ajuste del número de células presentes o para eliminar contactos incorrectos. El mecanismo más aceptado para explicar la muerte de neuronas en el desarrollo es que muchas células mueren al no recibir factores tróficos suplementados por las células blanco (Oppenheim, 1991; Johnson y Deckwerth, 1993; Voyvodic, 1996). A pesar de que la teoría neurotrófica es apoyada por muchos datos (ver Pittman y cols., 1994), recientemente se ha observado que también la actividad aferente, factores tróficos producidos por las células gliales y la matriz extracelular son factores que pueden contribuir a la supervivencia de algunas neuronas (Oppenheim, 1991).

Sin embargo, independientemente de los mecanismos de muerte neuronal durante el desarrollo del SN, vale la pena mencionar que existe una diferencia radical entre ésta y la que se ha observado en ciertas enfermedades o el envejecimiento: la primera, a pesar de eliminar tantas neuronas, en apariencia tiene consecuencias positivas y no negativas (o por lo menos no hemos sido capaces de detectar lo contrario), mientras que la neurodegeneración en organismos adultos

casi siempre tiene un impacto negativo sobre el funcionamiento del SN (Calne y cols., 1992). Existen muchos ejemplos de enfermedades neurodegenerativas agudas o crónicas, en las que la muerte neuronal parece ser la responsable de los déficits en funciones específicas, como la Corea de Huntington, la enfermedad de Alzheimer, el mal de Parkinson, la esclerosis lateral amiotrófica, la isquemia, la epilepsia, la demencia asociada al SIDA, etc. (Choi, 1988; Lipton y Rosenberg, 1994). Es por esta asociación entre disfunción y neurodegeneración en adultos que en los últimos años muchos investigadores han tratado de establecer cuáles son los caminos que llevan a una neurona a la muerte y si es posible evitar que ésto ocurra. El diseño de estrategias para evitar la muerte neuronal tiene una gran importancia, si se recuerda que las neuronas son células que han salido del ciclo celular y no pueden dividirse después de cierta etapa del desarrollo y que el funcionamiento del SN requiere del correcto establecimiento de redes celulares, constituidas de neuronas que deben estar en el lugar y tiempo correctos (Oppenheim, 1991). También resulta claro que el comprender mejor los mecanismos que conducen a la neurodegeneración, permitirá diseñar mejores formas de prevenir la muerte neuronal en el organismo adulto.

I.i. Tipos de muerte celular

El fenómeno de la muerte de una célula ha sido estudiado durante las últimas décadas, primero usando metodologías puramente descriptivas y posteriormente con disciplinas más dinámicas, especialmente la Bioquímica y la Biología Molecular. Es en el área de la Biología del Desarrollo donde se han

realizado un gran número de experimentos que arrojan resultados claros acerca de la muerte celular. Desafortunadamente, todavía nadie puede entender por qué algunas células mueren y otras sobreviven durante la formación de un organismo, pero se han sugerido posibilidades para explicar la muerte de ciertas poblaciones celulares, entre las que se encuentran las siguientes: 1) células que no tienen una función específica, y que parecieran vestigios evolutivos. 2) Células supernumerarias durante la formación de un órgano particular. 3) Células que tienen funciones iguales a otras. 4) Células que no se desarrollaron adecuadamente. 5) Células que han cumplido su función en la vida de un organismo y ya no son necesarias. 6) Células que serían potencialmente dañinas para el organismo (Ellis y cols., 1991).

En 1972, Kerr y cols. describieron las características morfológicas de varios tejidos que evidenciaban muerte celular, ya sea como resultado de la formación normal del organismo, o en algunas situaciones patológicas. Ellos empezaron a utilizar los términos necrosis y apoptosis (término griego que significa "caída de pétalos") para agrupar un conjunto de rasgos distintivos de una u otra condición. Cabe mencionar que actualmente apoptosis se usa como sinónimo de muerte celular programada (o muerte dirigida por genes), a pesar de que no son términos estrictamente equivalentes (Ellis y cols., 1991; Schwartz y cols., 1993; Martin y cols., 1994). No obstante que la apoptosis se refiere a un conjunto de rasgos morfológicos y bioquímicos, y que la muerte programada es el proceso que se inicia como respuesta a distintos estímulos, estos términos se han vuelto intercambiables debido a que

muchas de las células que mueren durante el desarrollo (es decir, mueren de acuerdo a un programa establecido), presentan características apoptóticas; en esta tesis se usarán ambos términos como sinónimos. Las observaciones asociadas a los tipos de muerte descritos por Kerr y cols., junto con otras que se han descrito más recientemente, se enumeran en la tabla 1.

Tabla 1. Características de apoptosis y necrosis*

<u>Apoptosis</u>	<u>Necrosis</u>
•Membrana celular y organelos íntegros	•Membrana celular y organelos dañados
•Cuerpos apoptóticos	•Membrana plasmática sin extrusiones
•Pérdida del volumen celular	•Hinchamiento
•Condensación de la cromatina	•Fragmentación nuclear inespecífica
•No se afectan células vecinas	•Daño en células contiguas
•Ruptura del DNA en fragmentos de 50-300 kb y después en múltiplos de 180 pb (escalera en geles)	•Degración del DNA en fragmentos que no presentan un patrón particular
•Proceso "activo" (muchas veces requiere síntesis de proteínas)	•Proceso "pasivo"
•Requiere energía	•Puede ocurrir sin energía
•Exposición de la fosfatidilserina a la cara extracelular de la membrana plasmática	•Exposición de la fosfatidilserina a la cara extracelular de la membrana, pero acompañada de daño membranal
•Liberación temprana de citocromo c de la mitocondria	

*Esta tabla se elaboró usando información de las siguientes referencias: Kerr y cols., 1972; Ellis y cols., 1991; Oppenheim, 1991; Johnson y Deckwerth, 1993; Schwartz y cols., 1993; Martin y cols., 1994; Kluck y cols., 1997; Yang y cols., 1997.

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Como se mencionó anteriormente, esta clasificación trató de establecer diferencias entre un tipo de muerte y otro, sin embargo, muchas veces resulta difícil, al observar un fenómeno, que éste llene todos los requerimientos para caer en una u otra categoría (Choi, 1996; Gwag y cols., 1997), y a veces un estímulo tóxico puede ocasionar apoptosis o necrosis, dependiendo de su severidad (Bonfoco y cols., 1995). Además, se han propuesto otras clasificaciones para los diferentes tipos de muerte celular (ver Oppenheim, 1991).

Actualmente, en general se acepta que todas las células poseen la maquinaria que eventualmente podría llevarlas a la muerte, la cual depende de un programa interno en la propia célula, aunque el medio extracelular puede ser el responsable de iniciar una de las varias cascadas de señales que convergen en la degeneración celular (Steller, 1995).

A continuación se detallarán algunos de los mecanismos involucrados en la muerte celular en dos modelos, que se aceptan como pertenecientes a apoptosis (o más correctamente muerte celular programada) y necrosis. En el primer caso, se describe el estado del conocimiento actual sobre los genes involucrados en la muerte celular observada durante el desarrollo del nemátodo *Caenorhabditis elegans*, sobre sus genes análogos en mamíferos, sobre otros genes de*Drosophila melanogaster* y de algunos virus que tienen influencia sobre esta ruta de suicidio. En el caso de la muerte necrótica, se describirán experimentos que han permitido establecer los mecanismos de la muerte neuronal producida por el glutamato (Glu) o sus análogos, particularmente en cultivos primarios.

I.ii. Muerte celular programada

Genes que promueven la apoptosis

La muerte celular que ocurre durante el desarrollo ha podido estudiarse en detalle en el nemátodo C. *elegans*, debido a que es un organismo formado por pocas células, lo cual ha permitido observar la degeneración celular *in vivo* y conocer muy bien a qué tiempo y cuáles células van a morir. De las 1090 células que se generan en un animal durante su desarrollo, 131 están destinadas a morir (105 de esas 131 son neuronas). En el estudio del proceso de eliminación de estas células, se han encontrado mutaciones espontáneas que han permitido establecer la participación de algunos genes en la supervivencia o muerte (Ellis y cols., 1991; Driscoll and Chalfie, 1992; Hengartner y Horvitz, 1994b; Steller, 1995). De esta manera, ha sido posible identificar genes que se requieren para la muerte celular, entre los que se encuentran *ced-3* y *ced-4*, puesto que mutantes que no poseen alguno de estos genes prácticamente no presentan muerte celular, dando lugar a organismos más grandes que los normales. Estos genes deben expresarse para que una célula degenere, puesto que en mosaicos genéticos, sólo las células que poseen *ced-3* funcional mueren. Es importante mencionar que en estos mutantes, las células que deberían morir son capaces de diferenciarse, y en general el organismo no tiene alteraciones a pesar de tener un mayor número de células.

Más recientemente, se ha podido establecer homología entre CED-3 (la proteína codificada por *ced-3*) y una familia de cisteína-proteasas que hidrolizan proteínas al encontrar aspartato (otra proteasa capaz de cortar después de aspartato es

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la granzima B, la cual es la responsable de la apoptosis inducida por linfocitos T citotóxicos). Estas proteasas de mamíferos, conocidas como caspasas, incluyen a la enzima convertidora de interleucina 1B (ICE por sus iniciales en inglés, caspasa 1), NEDD2, ICH1, ICH2 y CPP32 (caspasa 3) y se han agrupado en una familia que comparte en su sitio activo la secuencia de 5 aa glutamina-alanina-cisteína-arginina(glutamina)-glicina. Los sustratos hidrolizados por la acción de estas proteasas, y que podrían participar en el proceso de muerte, se cuentan: a) las propias caspasas, las cuales requieren de proteólisis para activarse; b) la polimerasa de poli-ADP-ribosa (PARP), que es una enzima que repara al DNA; c) la actina, sobre la que se ha hipotetizado que una vez fragmentada, es incapaz de unirse y así inactivar a la DNAsa I; d) GAS2, espectrina y fodrina, proteínas que forman parte del citoesqueleto; e) lámina nuclear B1, que podría contribuir a la condensación de la cromatina (Schwartz y Milligan, 1996). Por otra parte, la proteína CED-4 tiene un peso molecular de 63 kDa y no se ha podido establecer homología con ninguna otra proteína, por lo que no se conoce cuál puede ser su función (Yuan and Horvitz, 1992).

Genes que impiden la muerte celular

En *C. elegans* también se ha encontrado un gen cuya expresión impide la muerte: el *ced-9* (Ellis y cols., 1991), que en apariencia regula la acción de *ced-3* y *ced-4* (Hengartner y Horvitz, 1994b). El complejo panorama de las interacciones entre los genes *ced* se complica aún más con la observación reciente de que distintas formas de *ced-4* pueden promover (*ced-4S*) o proteger (*ced-4L*) de la muerte celular, y ambos

efectos son antagonizados por *ced-9* (Shaham y Horvitz, 1996). El gen *ced-9* tiene un homólogo en mamíferos, el proto-oncogen *bcl-2* (Hengartner y Horvitz, 1994a). BCL-2 es una proteína membranal de 26 kDa que se localiza en la mitocondria, el retículo endoplásmico (ER) y la membrana nuclear. Este polipéptido posee una serie de 19 aminoácidos (aa) hidrofóbicos que le sirven como señal para anclarse en la membrana por la porción carboxilo terminal, quedando la región amino terminal orientada hacia el citoplasma (Merry y Korsmeyer, 1997).

El *bcl-2* se clonó al encontrar el gen responsable de una gran proporción de ciertos linfomas en humanos, que resulta de una translocación entre los cromosomas 14 y 18 (Davies, 1995; Merry y Korsmeyer, 1997). Dicha translocación coloca a *bcl-2* bajo la influencia de un promotor muy activo de la síntesis de la cadena pesada de las inmunoglobulinas, lo que ocasiona una mayor expresión de BCL-2; la neoplasia entonces, es el resultado de que los linfocitos B sobreviven más tiempo del normal. Así, BCL-2 es el miembro prototipo de una familia surgida a partir de la comparación de la secuencia de distintas proteínas homólogas en los dominios designados como BH1, BH2 y BH3. Dentro de esta numerosa y creciente familia, hay genes que promueven la muerte celular, como *bax, bcl-xS, bad, bak* y *bik* y otros que por el contrario, protegen de la muerte como *bcl-2, bcl-xL* y *mcl-1* (Merry y Korsmeyer, 1997).

A continuación, describiré algunas de las características de BCL-2, por considerar que es una proteína muy interesante. La región carboxilo terminal de esta proteína no es esencial para su función protectora, sin embargo, los dominios BH1 y

BH2 parecen ser necesarios. Los residuos de glicina 145 en el dominio BH1 y triptofano 188 en el BH2 son indispensables para que BCL-2 se dimerice con miembros de su propia familia (Davies, 1995). La sobreexpresión de BCL-2, tanto *in vitro* como *in vivo*, ha dejado en claro que esta proteína juega un papel primordial en la supervivencia celular y neuronal. No obstante, su efecto protector no es universal, puesto que se han descrito algunos modelos en los que no es capaz de prevenir la muerte, sugiriendo que puede haber reguladores de su función. Entre los modelos en los que se ha demostrado su eficacia, se incluyen la prevención de la muerte inducida por la falta de factores de crecimiento, las radiaciones, condiciones oxidantes, excitotoxicidad e isquemia (Davies, 1995; Merry y Korsmeyer, 1997). La sobreexpresión de BCL-2 en neuronas de ratones transgénicos produjo que tales ratones presentaran un mayor número de neuronas en el SNC, lo que ocasionó hipertrofia cerebral. Al someter a estos ratones a isquemia permanente, se encontró una menor proporción de daño celular (Martinou y cols., 1994). En otro estudio con ratones que sobreexpresan BCL-2, esta proteína fue capaz de retrasar la aparición de esclerosis lateral amiotrófica (enfermedad caracterizada por la muerte de neuronas motoras) ocasionada por una mutación de la enzima superóxido dismutasa y prolongar el tiempo de vida de ratones (Kostic y cols., 1997).

En relación a los mecanismos mediante los cuales BCL-2 podría proteger a las células de la muerte, se han descrito los siguientes: disminuir los niveles de especies oxidantes en las células (Hockenbery y cols., 1993; Kane y cols., 1993), promover la homeostasis de calcio (Ca^{2+}) intracelular (Prehn y cols., 1994) y/o inhibir la liberación

del citocromo c de la mitocondria, lo cual parece ser un paso importante para desencadenar la muerte celular programada (Kluck y cols., 1997; Yang y cols., 1997). En estos últimos trabajos, queda claro que después de la liberación del holocitocromo c de la mitocondria, hay activación de caspasas (CPP32), degradación de proteínas blanco (ver figura 1), así como condensación nuclear y degradación del DNA en escalera, tanto en sistemas libres de células como en líneas celulares. También se establece que la despolarización de la mitocondria es posterior a la salida del citocromo c y que esta pérdida del potencial de membrana de la mitocondria es contrarrestada por BCL-2. En apariencia, es la fracción mitocondrial de BCL-2 la que impide la liberación del citocromo c. Es importante hacer hincapié en que, como en el caso de los genes *ced*, las complejas interacciones entre los miembros de la famila de *bcl-2* no están aún claras. En los próximos años podremos tener un panorama más completo de lo que sucede cuando una célula se encuentra en la disyuntiva de sobrevivir o morir.

Adicionalmente, se han descrito otros genes importantes en la muerte celular, los cuales se enlistan en la tabla 2, junto con los descritos en el texto.

Tabla 2. Genes y proteínas involucradas en la muerte o supervivencia

Gen o proteína	Origen	Comentario
ced-3, ced-4	C. elegans	Necesarios para la muerte celular programada
ICE, CPP32	Mamíferos	Proteasas promotoras de muerte celular. Equivalentes a CED-3
ced-9	C. elegans	Regulados regativo de *ced-3* y *ced-4*
bcl-2	Mamíferos	Equivalente a *ced-9*

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ces-1	*C. elegans*	Necesario para la muerte de neuronas faríngeas
ces-2	*C. elegans*	Regulador negativo de *ces-1* . Miembro de la subfamilia PAR (ricas en prolina y aa ácidos) de factores de transcripción bZIP
deg-1, mec-4	*C. elegans*	Mutaciones dominantes de estos genes promueven muerte celular caracterizada por hinchamiento. Esta vía parece ser independiente de *ced-3* y *ced-4*. Todas las mutaciones descritas carecen de una alanina en la region que teoricamente es un dominio transmembranal. Aunque no se conoce la función de estas proteínas, se ha hipotetizado que pueden ser canales iónicos o transportadores membranales debido al tipo de muerte observada
p35	Baculovirus	Protege de la muerte en insectos, nemátodos y mamíferos. Inhibe miembros específicos de la familia de las caspasas
crmA	Baculovirus	Previene la muerte celular. Acción similar a la de p35
IAP	Baculovirus	Inhibidor de apoptosis
reaper	*D. melanogaster*	Codifica para un polipéptido de 65 aa y promueve la muerte celular programada sin participar activamente en el proceso. Podría actuar al activar la cascada de CED-3/ICE, o bloqueando la acción de CED-9/BCL-2
E1B19K	Adenovirus	Proteína protectora contra muerte. Comparte homología con los dominios BH1 y BH2 de BCL-2
p53	Mamíferos	Es una fosfoproteína que se localiza en el núcleo y que podría ser un factor de transcripción. Se activa por la presencia de DNA de una hebra o dañado. Puede hechar a

| | | andar el mecanismo de muerte que es susceptible de ser bloqueado por BCL-2 |
| *c-myc* | Mamíferos | La proteína codificada por este gen es presumiblemente un factor de transcripción que juega un papel dual: por un lado estimula la proliferación y por otro, en algunos casos su presencia promueve la apoptosis |

Recientemente ha surgido una teoría que trata de establecer una asociación entre los fenómenos de división celular y muerte, basada en que muchos de los oncogenes (los cuales inducen la proliferación celular) tienen una participación importante en el proceso de muerte (Heintz, 1993) y en la morfología que presentan neuronas cultivadas a las que se las ha retirado el factor de crecimiento nervioso. Estos datos se ven apoyados por la observación de que en ratones transgénicos que expresan el antígeno T del virus SV40 en el cerebelo, al inducir la proliferación, se observó neurodegeneración (Pittman y cols., 1994). Así, la muerte celular genéticamente dirigida podría ser resultado de un intento fallido de proliferación de las neuronas posmitóticas.
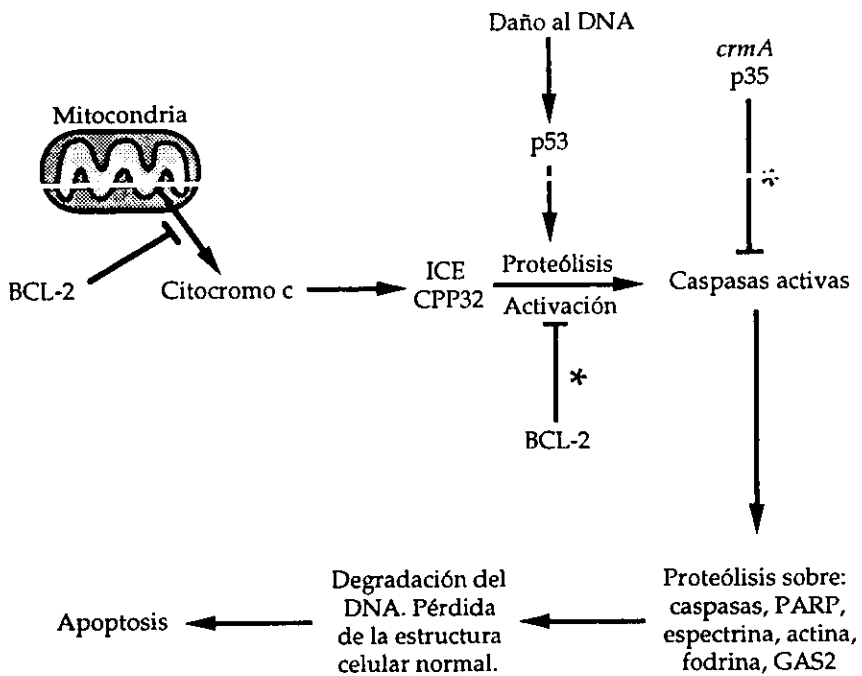
Figura 1. Descripción de la secuencia que lleva a la muerte celular programada y en qué punto se cree actúan los inhibidores de este proceso. Las caspasas se activan al sufrir proteólisis, dando como resultado dos péptidos. Estos dos fragmentos deben dimerizarse para formar la caspasa activa. * señala un bloqueo del proceso. Sólo se representan genes y proteínas relevantes para los mamíferos. Las abreviaturas se definen en la página iii y la tabla 2.

I.iii. Excitotoxicidad

Como se mencionó antes, existen muchas enfermedades en las que se piensa que la muerte neuronal es la responsable de los síntomas clínicos. Recientemente, se ha analizado el papel del Glu en la muerte neuronal asociada con algunos procesos degenerativos agudos y crónicos.

El término excitotoxicidad trata de asociar las propiedades despolarizantes (excitadoras) del Glu sobre neuronas, con su capacidad de inducir neurodegeneración. A pesar de que la observación de que el Glu podía causar muerte neuronal se hizo *in vivo* (Olney, 1969), es en sistemas *in vitro* dónde se han establecido más claramente los cambios relacionados con la excitotoxicidad, por lo que los experimentos que relataré a continuación fueron hechos primordialmente en cultivos neuronales primarios. Es pertinente resaltar que en la actualidad se piensa que muchas enfermedades del SN central (SNC) se deben a la excitotoxicidad por Glu, y también es claro que el Glu participa en funciones normales del cerebro como la cognición, la memoria, el movimiento y las sensaciones. En el caso de las patologías, se cree que la concentración extracelular de Glu se eleva ya sea por un aumento en la liberación de este neurotransmisor, al disminuir su recaptura luego de su liberación, o bien al liberarse de células que han muerto (Choi, 1988; Lipton y Rosenberg, 1994).

Receptores a Glu

El Glu es capaz de activar a dos grupos de receptores presentes en neuronas, los que permiten el paso de iones hacia dentro de la célula (ionotrópicos) y los que

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están acoplados a un sistema de segundos mensajeros (metabotrópicos). Dentro de los receptores ionotrópicos se encuentran los receptores que reconocen al N-metil-D-aspartato (NMDA) y los que reconocen al ácido α-amino-3-hidroxi-5-metil-4-isoxazol propiónico AMPA o al kainato (no-NMDA). A diferencia de los canales de potasio sensibles a voltaje, que son tetrámeros, los receptores ionotrópicos a Glu parecen estar formados por 5 subunidades. Al activarse, los receptores NMDA permiten una entrada lenta de Na^+, K^+, y Ca^{2+} a las células. Este receptor, distribuido ampliamente en el SNC, tiene varios sitios que modulan la actividad del canal (sitios para magnesio, glicina, poliaminas, zinc). La clonación de la subunidad 1 del receptor a NMDA (NMDAR1, Moriyoshi y cols., 1991) permitió establecer que ésta es suficiente para formar receptores funcionales con todas las características del receptor nativo, aunque con corrientes iónicas de menor magnitud, lo que sugiere la presencia de subunidades accesorias *in vivo*. Se han descrito 7 isoformas del NMDAR1 producto de "splicing" alternativo y se predice, por su secuencia de aa, que este receptor posee cuatro dominios transmembranales con un extremo amino terminal extracelular muy grande. Más adelante se describieron 4 isoformas del NMDAR2, las cuales no forman canales funcionales por sí solas, pero potencian la actividad del NMDAR1 (Nakanishi, 1992). Por su parte, los receptores AMPA/kainato permiten el paso de sodio y son los responsables de la despolarización rápida de las neuronas postsinápticas. Los receptores no-NMDA son parecidos a los NMDA en sus motivos estructurales (4 segmentos que atraviesan la membrana y una porción extracelular grande, correspondiente al extremo amino terminal). Al principio se pensó que estos

receptores eran muy poco permeables al Ca^{2+}, pero la clonación de distintas subunidades que se expresaron en ovocitos de *Xenopus laevis*, permitió establecer que hay subunidades que permean Ca^{2+} (GluR1, GluR3). Sin embargo, hay una subunidad (GluR2) que al ser coexpresada con otras tiene un efecto dominante negativo sobre el paso de Ca^{2+} a través del canal (es decir, cuando GluR2 está presente, el Ca^{2+} no entra a las células al activarse el receptor; Hollman y cols., 1991). Los cDNAs de los receptores GluR2 y GluR6 codifican polipéptidos que tienen una arginina en el segundo dominio transmembranal (el que se piensa forma el poro iónico) en el sitio donde los demás receptores no-NMDA tienen una glutamina. Sin embargo, la secuencia genómica de GluR2 reveló que en esa posición se debería encontrar una glutamina, por lo que se propuso que el mRNA de esta subunidad es editado en su totalidad, mientras que otras subunidades son modificadas parcialmente (GluR5 y GluR6) y otras no sufren ningún cambio (GluR1, GluR3, GluR4; Gasic y Heinemann, 1992).

La activación de los receptores metabotrópicos glutamatérgicos puede conducir a la producción de inositol trifosfato (IP_3), o bien a la inhibición de la adenilato ciclasa. Se han descrito 8 tipos de receptores metabotrópicos a Glu, los cuales tienen los 7 dominios transmembranales característicos de los receptores acoplados a proteínas G. A pesar de que la homología de los receptores a Glu acoplados a segundos mensajeros con otros receptores metabotrópicos es mayor a 40%, los primeros poseen un gran extremo amino terminal característico hacia fuera de la célula. Evidentemente, la respuesta a la activación de estos receptores por Glu

no es tan rápida como la de los receptores ionotrópicos. El papel neurofisiológico de los receptores metabotrópicos se ha empezado a estudiar recientemente y en apariencia, su activación lleva a una reducción en la liberación de neurotransmisores (Nakanishi, 1992, 1994, ver figura 2).

Degeneración neuronal producida por Glu en cultivos primarios

Experimentos hechos por el grupo de Choi en la década pasada (Choi, 1987; Choi y cols.. 1987), usando cultivos de corteza cerebral de ratón han permitido establecer inequívocamente que el Glu puede causar muerte neuronal. Los primeros trabajos se enfocaron a describir los cambios provocados por el Glu (0.5-1 mM durante 5 minutos) en las neuronas cultivadas. Dichas alteraciones fueron dividas en dos fases: una inicial en donde se observó hinchamiento debido a la entrada de sodio y cloruro a las neuronas y una segunda fase, en donde el daño celular era mucho más evidente, que dependía de la entrada de Ca^{2+}. Esta correlación entre la entrada de Ca^{2+} y la degeneración celular fue confirmada más tarde, y se pudo identificar un aumento inicial en el Ca^{2+} intracelular ($[Ca^{2+}]_i$) debido a la acción despolarizante del Glu. Este incremento inicial es contrarrestado por los sistemas intracelulares de recaptura de Ca^{2+} citoplásmico; un aumento posterior, el cual no puede ser corregido se asoció con la muerte de las neuronas (Randall y Thayer, 1992). Más aún, la proporción de neuronas muertas correlacionó bien con la captura de $^{45}Ca^{2+}$ por las células (Hartley y cols., 1993) y la introducción de quelantes de Ca^{2+} a las células atenuó la toxicidad por Glu (Tymianski y cols., 1994). Es importante recalcar que esta entrada de Ca^{2+} se atribuyó a la activación del receptor NMDA y no a

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los canales de Ca^{2+} sensibles a voltaje (VSCC, ver artículo 1).

Además, se estableció que los antagonistas del receptor NMDA protegían de la muerte neuronal (Choi y cols., 1988). En contraste, la activación de los receptores metabotrópicos a Glu protege contra la excitotoxicidad por NMDA (Koh y cols., 1991; Bruno y cols., 1997). Aunque después se describió que los agonistas no-NMDA también eran capaces de inducir muerte (con tiempos de exposición mucho mayores comparados con los que se requerían al adicionar NMDA; Koh y cols., 1990), en general se acepta que el receptor NMDA está más relacionado con la neurodegeneración. De hecho, cultivos corticales preparados de cerebros de ratones que no expresan el NMDAR1 no son afectados por Glu (Tokita y cols., 1996). Los efectos que tiene la elevación de la $[Ca^{2+}]_i$ incluyen la activación de proteasas (las cuales pueden actuar sobre proteínas del citoesqueleto), lipasas y endonucleasas, lo que indudablemente contribuye a la muerte de las neuronas (ver abajo y figura 2).

Participación de especies oxidantes en el daño neuronal por Glu

Una teoría que se ha relacionado mucho con la excitotoxicidad es el daño mediado por especies reactivas de oxígeno (ROS). De las moléculas que se forman dentro de la célula y que son ROS, el radical hidroxilo ($\cdot$OH, dónde el punto representa un electrón sin aparear) es el más reactivo y por lo tanto el que puede causar mayores alteraciones en los componentes celulares. Se piensa que los efectos oxidantes de las ROS sobre los lípidos, el DNA y las proteínas contribuyen al daño celular. Aunque normalmente las células poseen sistemas para eliminar a las ROS, que consisten en la superóxido ($\cdot O_2^-$) dismutasa, que transforma al $\cdot O_2^-$ en peróxido

de hidrógeno (H_2O_2) y las enzimas catalasa y glutatión peroxidasa, que se encargan de transformar al H_2O_2. Estos sistemas en muchas ocasiones no son suficientes para eliminar a las ROS.

Las fuentes de las ROS en la célula son: 1) la formación de $\cdot O_2^-$ y H_2O_2 a consecuencia del metabolismo oxidativo de las células nerviosas; a este respecto, se ha demostrado que en mitocondrias aisladas de cerebro una elevación en el Ca^{2+} o el Na^+ en el medio puede conducir a la generación de radicales libres ($\cdot OH$, Dykens, 1994); 2) enzimas como la monoamino oxidasa, la tirosina hidroxidasa y la L-amino oxidasa producen H_2O_2; 3) la activación de fosfolipasa A_2 por Ca^{2+} produce ácido araquidónico, que genera $\cdot O_2^-$ al ser metabolizado; el ácido araquidónico además puede promover la liberación de Glu e inhibir su recaptura; 4) la activación por Ca^{2+} de la sintasa del óxido nítrico (NOS) genera óxido nítrico (NO), que es capaz de reaccionar con $\cdot O_2^-$ para formar peroxinitrito (ONOOH), que es una fuente importante de $\cdot OH$; el NO es capaz de difundir a otras células, propagando así la oxidación; 5) la xantina deshidrogenasa es transformada en xantina oxidasa por proteasas activadas por Ca^{2+}; la oxidación de la xantina genera ácido úrico, $\cdot O_2^-$ y H_2O_2; 6) la reacción de Fenton, que consiste en la oxidación de un metal (generalmente fierro) y la descomposición de H_2O_2 en $\cdot OH$ y OH^- (Olanow, 1993; Coyle y Puttfarcken, 1993; Cohen, 1994). Para enfermedades crónicas del SNC que presentan neurodegeneración, se ha propuesto que el daño oxidativo debido a la actividad de las mitocondrias podría jugar un papel importante en la patogénesis (Shigenaga y cols., 1994).

<u>Papel de la recaptura de Glu en la excitotoxicidad</u>

Me parece pertinente mencionar que cuando se realizaron los primeros experimentos de excitotoxicidad en cultivos, parecía que la participación de la recaptura de Glu no era crucial para la neurodegeneración (Choi y cols., 1987). No obstante, después se observó que al impedir la entrada de Glu a las células se podía potenciar el efecto tóxico de Glu añadido a cultivos (Robinson y cols., 1993), o bien causar muerte neuronal al impedir la recaptura de Glu durante 1-2 semanas en cultivos organotípicos de médula espinal (Rothstein y cols., 1993). Apoyando la participación de los sistemas de recaptura de Glu en la excitotoxicidad, fue posible demostrar que se podía inducir neurodegeneración con la acumulación de Glu endógeno de los cultivos, si la inhibición de los transportadores para Glu se mantiene durante algunas horas (Velasco y cols., 1996). Aún más, al eliminar uno de los transportadores de Glu presente en las células gliales en ratones transgénicos, se observó que estos animales presentaban epilepsia, neurodegeneración en el hipocampo y una mayor susceptibilidad a daño en la corteza (Tanaka y cols., 1997).

Algunos de los mecanismos involucrados en la muerte neuronal por Glu se representan en la figura 2, sin embargo, hay acciones del Glu sobre componentes neuronales que previenen la muerte celular, como se mencionará mas adelante. En el esquema se representa una sinapsis glutamatérgica activa: al liberar el Glu, se activan los receptores ionotrópicos y metabotrópicos y el neurotransmisor es después capturado por los transportadores de alta afinidad (neuronales o gliales). Como se describió arriba, la activación de los receptores NMDA y no-NMDA lleva a la

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despolarización y a la entrada de Ca^{2+} a la neurona, lo que conduce al hinchamiento, la activación de enzimas potencialmente dañinas, y la generación de ROS. Cabe resaltar el hecho de que la muerte neuronal se acompaña de la liberación inespecífica de Glu al medio, lo cual se ha propuesto, propaga la acción tóxica del Glu. La activación de los receptores metabotrópicos, en contraposición de lo que ocurre con los receptores ionotrópicos a Glu, puede proteger de la muerte neuronal al impedir la propagación de la excitación al disminuir la liberación sináptica de Glu y/o proteger de la muerte por un mecanismo desconocido en la neurona postsináptica. También se ha sugerido que el NO puede tener un efecto protector dependiendo del estado de óxido-reducción de la célula, al disminuir la actividad del receptor NMDA (Lipton y cols., 1993).
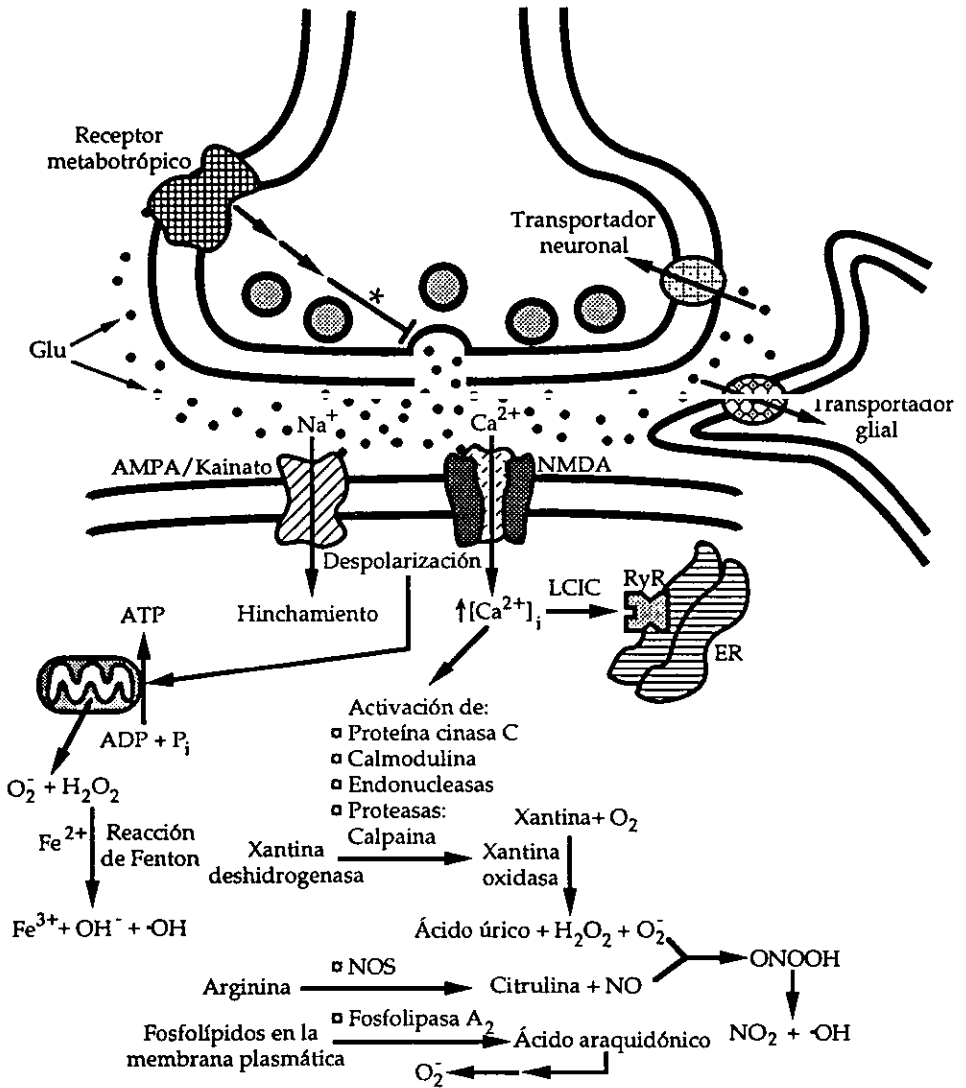
Figura 2. Procesos asociados con la excitotoxicidad por Glu. 2 flechas sucesivas indican pasos intermedios. En la presinapsis, * señala un bloqueo de la liberación de Glu. Las abreviaturas se definen en la página iii.

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II. Rojo de rutenio

En esta tesis se utilizó al rojo de rutenio (RR) como una toxina selectiva para neuronas y el modelo que se utilizó fue el de cultivos primarios de neuronas y astrocitos aislados del SNC de la rata. A continuación se revisa la literatura en relación a las propiedades del RR y sus efectos sobre sistemas biológicos.

II.i. Generalidades

El RR es un colorante inorgánico, sintético y policatiónico que se ha utilizado en microscopía electrónica porque forma, junto con el tetraóxido de osmio un complejo con carga positiva que es opaco a los electrones. Su fórmula es la siguiente:

$$[(NH_3)_5 \; Ru^{III}\text{-}O\text{-}(NH_3)_4 \; Ru^{IV}\text{-}O\text{-}Ru^{III}(NH_3)_5]^{6+} \; 6 \; Cl^-$$

Debido a su carácter catiónico, se ha descrito que tiene interacción con muchos componentes celulares de tipo aniónico (Luft, 1971). Estudios de cristalografía con RR (Carrondo y cols., 1980) han permitido establecer que la molécula tiene un eje central formado por los enlaces N-Ru-O-Ru-O-Ru-N, que el Ru se encuentra en el centro de un octaedro y que los enlaces entre el Ru y el N son equivalentes en cuanto a su longitud. De esta manera, el RR en solución sería un cilindro con 1.17 nm de altura y 0.5 nm de diámetro. Se ha calculado que el diámetro de esta molécula es de 1.13 nm.

II.ii. Efectos del RR sobre la mitocondria y el retículo endoplásmico

Este compuesto se ha asociado con el catión Ca^{2+} desde las primeras descripciones de sus efectos sobre sistemas biológicos, en los años setentas. El primero de estos estudios lo situó como un potente inhibidor de la captura de Ca^{2+} dependiente de energía por la mitocondria (Moore, 1971). Estudios posteriores más

extensos confirmaron que a concentraciones nM y μM, el RR inhibía de manera muy importante la entrada de Ca^{2+} a las mitocondrias hepáticas (Vasington y cols., 1972; Reed y Bygrave, 1974; Luthra y Olson, 1977; Sparagna y cols., 1995) y establecieron que el RR puede inhibir de manera no competitiva la unión de Ca^{2+} a la mitocondria (Vasington y cols., 1972; Reed y Bygrave, 1974), además de disminuir la respiración e inhibir la actividad de la ATPasa mitocondrial a concentraciones de 10 a 100 μM (Vasington y cols., 1972).

Posteriormente, el RR se empezó a utilizar como inhibidor de la salida de Ca^{2+} de otros organelos que, además de la mitocondria, participan en la remoción de Ca^{2+} citoplasmático cuando la concentración de este catión divalente aumenta, como el sarcolema de músculo esquelético y el retículo sarcoplásmico (SR) de músculo esquelético o cardíaco. El RR a una concentración >20 μM se unió al sarcolema aislado y así bloqueó la unión de Ca^{2+} casi completamente. Esta inhibición se redujo ligeramente al extraer los lípidos de la preparación. Adicionalmente, el RR a una concentración de 100 μM produjo una disminución de aproximadamente el 50% de la actividad de la ATPasa del sarcolema (Madeira y Antunes-Madeira, 1974).

En el SR de músculo se han identificado por lo menos 2 tipos de receptores que son responsables de la salida de Ca^{2+} necesaria para la contracción muscular; éstos son los receptores para IP_3 y para el alcaloide ryanodina (RyR). En el caso del RyR, se han aislado los cDNAs para 2 proteínas, una que se encuentra principalmente en músculo esquelético y otra que se localiza en el corazón y que son 66% homólogas entre sí. Este receptor se activa por la entrada de Ca^{2+} (fenómeno

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conocido como liberación de Ca^{2+} inducida por Ca^{2+}, LCIC) o por cafeína. El RyR del tipo de músculo cardíaco es el más abundante en el cerebro (Kuwajima y cols., 1992). En el SR cardíaco (Chamberlain y cols., 1984) y de músculo esquelético (Palade, 1987), el RR inhibió la LCIC y la acción de la cafeína a concentraciones en el intervalo nM. En contraste, en una preparación cardíaca de fibras musculares de aves, el RR (3 µM) fue capaz de inhibir tanto la salida de Ca^{2+} inducida por IP_3 como la LCIC, pero sorprendentemente potenció la salida de Ca^{2+} inducida por cafeína en concentraciones menores a 10 µM. Este aumento en la salida de Ca^{2+} al agregar la cafeína no se observó con concentraciones mayores de RR. De hecho, con RR 100 µM hubo una inhibición del 20% en el aumento del $[Ca^{2+}]_i$ al adicionar cafeína. Todos estos efectos del RR fueron duraderos e incluso se potenciaron al dejar transcurrir más tiempo (Vites y Pappano, 1992, 1994).

Evidencias adicionales de la interacción del RR con el RyR se produjo al estudiar la unión de ryanodina en presencia de RR en fracciones microsomales de cerebro y cerebelo. En este estudio, el RR 10 µM produjo inhibiciones del 65-75% de la unión de ryanodina, mientras que la concentración inhibitoria al 50% (IC_{50}) para la liberación de Ca^{2+} estimulada por cafeína fue de 40 µM para cerebro y >60 µM para el cerebelo (Mészaros y Volpe, 1991). Incluso en fracciones microsomales obtenidas de neuronas granulares de cerebelo en cultivo, el RR puede impedir la unión de ryanodina con una $IC_{50}=1.7$ µM (Rosa y cols., 1997). Para finalizar con la interacción del RR con el SR cardíaco, debo mencionar que el RR (30 µM) también es capaz de diminuir 80% la fosforilación del RyR por calmodulina, mientras que la ryanodina

promueve la adición de fosfato a dicha proteína. Estas acciones están relacionadas directamente con las capacidades del colorante para inhibir la salida de Ca^{2+} y del alcaloide de liberar Ca^{2+}, por lo que se ha sugerido que al interaccionar con el RyR, el RR impide la fosforilación de la proteína, lo cual en apariencia es necesario para la salida de Ca^{2+} del SR (Netticadan y cols., 1996).

Recientemente se ha establecido que el RR se une al RyR de músculo esquelético en los sitios (5 en total) del receptor que unen Ca^{2+} (Chen y MacLennan, 1994). Al incorporar este mismo receptor a bicapas lipídicas, Ma (1993) pudo observar una inhibición del paso de Ca^{2+} a través del RyR al adicionar concentraciones nanomolares de RR. La concentración de RR necesaria para observar la inhibición fue menor a voltajes más positivos, lo que sugiere que la entrada de RR al canal hace más eficaz su acción. Además, se encontró que el RR era capaz de inhibir al RyR cuando se aplica tanto en el lado mioplásmico como en el lumen del SR, aunque en el primer caso se encontró un número de Hill cercano a 2 y en el segundo muy próximo a 1.

La visión general de los experimentos en el SR es la siguiente: el RR inhibe eficazmente la LCIC y la salida de Ca^{2+} debida a IP_3. Sin embargo, a pesar de que la salida de Ca^{2+} inducida por cafeína, al igual que la LCIC se da a través del RyR, al parecer las concentraciones de RR que pueden inhibir la liberación de Ca^{2+} por cafeína son 2 o 3 órdenes de magnitud mayores que las que previenen la LCIC, lo que sugiere que el RR está bloqueando más eficientemente el sitio del RyR que detecta una elevación en la $[Ca^{2+}]_i$ que el sitio del RyR que une cafeína.

II.iii. Acciones del RR sobre algunas proteínas

Como se mencionó en la sección anterior, el RR es capaz de inhibir la función de la ATPasa de Ca^{2+} de la mitocondria y el sarcolema. En el SR esta proteína es la responsable del transporte activo de Ca^{2+} hacia dentro del retículo, favoreciendo así la relajación muscular, y tiene 2 sitios cooperativos de unión para Ca^{2+}. Walter y de Meis (1986) describieron el efecto del RR sobre esta ATPasa. Estos autores, usando concentraciones de RR hasta 200 µM, no observaron inhibición del transporte de Ca^{2+} debido a la ATPasa, pero describieron una inhibición por RR 20 µM de la reacción reversa (esto es, la síntesis de ATP acoplada al establecimiento del gradiente de Ca^{2+}), y por RR 200 µM de la salida de Ca^{2+} inducida por arsenato. Usando el mismo sistema, Corbalán-García y cols. (1992) localizaron dos sitios de unión a RR en la ATPasa, con valores de Km de 4.5 µM y 2 mM. Estos sitios en apariencia no son los mismos de unión para Ca^{2+}, pero la unión de RR a la proteína impide la interacción con Ca^{2+}, e incluso el colorante a una concentración de 100 µM desplaza casi completamente al Ca^{2+} unido a la ATPasa.

Por otra parte, y tomando como referencia las interacciones del RR con los sitios que unen Ca^{2+}, Charuk y cols. (1990) encontraron que el colorante no sólo era capaz de inhibir la unión del Ca^{2+} (K_i=72 µM) a la calsecuestrina, sino que se unía a la calmodulina, a la ATPasa de Ca^{2+} del SR, a la troponina C y a la proteína S-100 de cerebro, todas proteínas con sitios de unión a Ca^{2+}. Esta interacción con RR se realizó con dichas proteínas transferidas a nitrocelulosa y la detección de los polipéptidos fue tan eficaz como la autorradiografía con $^{45}Ca^{2+}$, por lo que los autores propusieron

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usar al RR para identificar de manera fácil proteínas que interaccionan con Ca^{2+}. Más adelante, Sasaki y cols. (1992) estudiaron la interacción del RR con la calmodulina, proteína que al unir Ca^{2+} cambia de conformación y activa a otras proteínas al unirse a ellas. El RR pudo inhibir la activación por calmodulina de una cinasa de miosina, presumiblemente al interferir con la unión de Ca^{2+} a la calmodulina. La IC_{50} para este efecto fue de 18.5 μM (en presencia de Ca^{2+} 0.1 mM) usando las proteínas aisladas. En este mismo trabajo, se pudo observar también la inhibición de la calmodulina por RR en un modelo de contracción de tejido vascular.

Otro efecto interesante del RR es la inhibición de la polimerización *in vitro* de la tubulina de cerebro a concentraciones equimolares. El colorante fue incluso capaz de promover la despolimerización de microtúbulos formados. Más aún, el RR a una concentración de 400 μM disminuyó 40% el transporte de leucina, que se da a través de microtúbulos, en el nervio ciático de la rana permeabilizado con tritón X-100 (Deinum y cols., 1981). El sitio de unión de RR a tubulina, en contraste con lo anteriormente descrito, no está relacionado con el sitio de unión de Ca^{2+} a la tubulina (Deinum y cols., 1985).

De los experimentos descritos en este apartado puede decirse lo siguiente: al igual que en el SR y la mitocondria, el RR interacciona con los sitios de unión a Ca^{2+} de las proteínas (excepto en el caso de la tubulina). Sin embargo, las concentraciones de RR necesarias para observar la inhibición de la unión de Ca^{2+} son mayores en el caso de las proteínas aisladas que en los organelos, lo que podría deberse a que las proteínas presentes en las membranas de los organelos tengan sitios de mayor

afinidad por el RR o bien que el ambiente lipídico permita una interacción más eficaz entre las proteínas y el RR.

II.iv. Efecto del RR sobre la liberación de neurotransmisores

La entrada de Ca^{2+} a la terminal presináptica es el evento que desencadena la liberación de neurotransmisores. El paso de este catión a través de la membrana se da a través de VSCC. Estos canales están constituidos de distintas subunidades: α_1, α_2/δ, β y en algunos casos γ. La subunidad que contiene el canal y el sensor de voltaje es la α_1, que tiene una masa molecular de 212-230 kDa. Este polipéptido incluye 4 dominios homólogos que contienen cada uno 6 segmentos transmembranales. Entre los segmentos transmembranales 5 y 6 se encuentra una asa que presumiblemente forma el poro y el segmento transmembranal 4, que contiene aa cargados positivamente, es el responsable de detectar los cambios en el voltaje membranal (McCleskey, 1994; De Waard y cols., 1996). De acuerdo a sus características fisiológicas y farmacológicas, los VSCC se han clasificado en 5 grupos (Spedding y Paoletti, 1992; De Waard y cols., 1996), los cuales se describen en la tabla 3.

Tabla 3. Clasificación y características de los VSCC

Tipo	$V_{apertura}$	$t_{inactivación}$	Antagonistas	Localización
L	-30 mV	>500 ms	Dihidropiridinas	Neuronas, músculo
N	-30 mV	50 - >500 ms	ω-conotoxina	Neuronas
T	-70 mV	10-80 ms	Flunarizina (?)	Neuronas, músculo
P	-40 mV	>500 ms	ω-agatoxina IVA	Neuronas

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| R | -40 mV | 20-40 ms | ----- | Neuronas |

Hasta el momento, todos los datos apuntan a que el RR bloquea preferencialmente los VSCC tipo N y P (Massieu y Tapia, 1988; Hamilton y Lundy, 1995). Hasta el momento no se ha podido establecer el sitio de los VSCC con el que interactúa el RR.

Los efectos del RR sobre la liberación de neurotransmisores, en animales a los que se les administró sistémicamente el colorante, así como las alteraciones observadas al inyectar RR en distintas regiones del cerebro se describen y discuten en el artículo "Ruthenium red as a tool to study calcium channels, neuronal death and the function of neural pathways" (artículo 1).

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COMMENTARY

RUTHENIUM RED AS A TOOL TO STUDY CALCIUM CHANNELS, NEURONAL DEATH AND THE FUNCTION OF NEURAL PATHWAYS

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Abstract—The inorganic polycationic dye ruthenium red (RuR) exerts several effects on the nervous system when added in physiological solutions, both *in vivo* and *in vitro*. Part of these effects, including the paralysis observed in mammals after the systemic administration of RuR, can be accounted for by the binding of RuR to nerve ending membranes, which results in inhibition of Ca^{2+} influx through voltage-sensitive calcium channels and the consequent inhibition of neurotransmitter release. On the other hand, the administration of RuR into the cerebrospinal fluid induces intense convulsive activity, and its microinjection into the substantia nigra reticulata or the hippocampus leads to various motor behavior alterations that can be related to hyperexcitability of the neurons of the injected region. In addition, RuR penetrates the neuronal somata present in the area injected and induces cell destruction, which has been interpreted as an excitotoxic action of the dye. The penetration and the toxicity of RuR were also observed in primary neuronal cultures but did not occur in pure glial cultures, suggesting a selective action on neurons. In the present article the *in vitro* and *in vivo* effects of RuR are reviewed and discussed in terms of the usefulness of the dye as an interesting tool to study calcium channels linked to transmitter release, neuronal death mechanisms and the function of neural pathways. Copyright © 1996 Elsevier Science Ltd

Calcium ions play a fundamental role in the physiology of interneuronal communication and in the mechanisms of neuronal cell death. It has been established, both in central and peripheral synapses, that the presynaptic membrane possesses voltage-sensitive calcium channels (VSCC), which are opened by depolarization, and that the influx of Ca^{2+} through these channels results in an increase in its cytoplasmic concentration, which triggers the exocytotic release of neurotransmitters. This Ca^{2+} increase, however, is spatially restricted, transient and limited in amount, owing to the efficient intracellular Ca^{2+}-buffering mechanisms, which, together with plasma membrane Ca^{2+}-ATPases and Na^{+}–Ca^{2+} exchangers, are responsible for maintaining a submicromolar intracellular concentration of the cation. Such a low concentration has to be kept against a concentration gradient of about 10,000 (approximately 10^{-7} versus 10^{-3} M).

Besides the VSCC, Ca^{2+} may enter the neuron through channels associated with neurotransmitter receptors, which are permeable to this cation and generally also to Na^{+}. The main receptors of this kind in the central nervous system (CNS) are the different types of excitatory amino acid receptor, including those recognizing *N*-methyl-D-aspartate (NMDA), kainic acid and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). Overactivation of these receptors, particularly the NMDA type, results in a massive entrance of Ca^{2+} that eventually leads to neuronal cell death. In fact, the exposure to a glutamate excess in the extracellular medium is extremely neurotoxic and such neurotoxicity is prevented by antagonists of the NMDA receptor, whereas no damage by Ca^{2+} entering through VSCC has been described (Choi, 1988; Hartley *et al.*, 1993; Tymianski *et al.*, 1993; Siesjö, 1994). Furthermore, it is believed that this type of receptor is involved in the elevation of intracellular Ca^{2+}, occurring as a delayed effect of ischemia or anoxia of cerebral tissue, which in turn seems to be involved in neuronal death (Meldrum and

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Garthwaite, 1990; Manev *et al.*, 1990; Siesjö, 1994). Other sources of cytoplasmic Ca^{2+} are the intracellular organelles responsible for its sequestration, such as the endoplasmic reticulum or the mitochondria. An increase in Ca^{2+} occurs when these buffering mechanisms are blocked or when an activation of the $Na^+–Ca^{2+}$ exchange at intracellular membranes is induced by augmented intracellular Na^+ concentrations (Mody and MacDonald, 1995; Simpson *et al.*, 1995). The neuronal destruction observed after a sustained elevation of cytoplasmic Ca^{2+} is due to a variety of factors, such as alterations of mitochondrial function, disruption of cytoskeleton organization, production of free radicals, membrane damage and activation of proteases (Mattson, 1994; Siesjö, 1994).

The foregoing notions imply that drugs capable of interacting with the intracellular or plasma neuronal membranes, in such a way that Ca^{2+} transport is modified, should affect neurotransmitter release and/or induce neuronal death. Drugs blocking VSCC should inhibit neurotransmitter release, whereas those inducing Ca^{2+} entry should stimulate it. Additionally, the latter could produce neuronal destruction, depending on the duration of the effect, the nature of its mechanism and the cellular site at which Ca^{2+} entry occurs.

Several types of VSCC have been described, which are differentially affected by various blockers and differ also in voltage sensitivity, speed of opening and closing and possible membranal location along the neuron (Spedding and Paoletti, 1992; Siesjö, 1994; Olivera *et al.*, 1994; Tareilus and Breer, 1995). Thus, it is possible to distinguish pharmacologically between the VSCC directly related to the cytoplasmic pool of Ca^{2+} involved in neurotransmitter release from those unrelated to this process, because only the drugs blocking the former channels should also inhibit the Ca^{2+}-dependent release. Among the compounds that seem to block Ca^{2+} movements through any of the VSCC types are divalent cations such as Mg^{2+}, Mn^{2+} and Co^{2+}, the trivalent cation La^{3+}, and several organic compounds, including verapamil, methoxyverapamil, and substituted 1,4-dihydropyridines such as nifedipine, nitrendipine and nimodipine. Other important blockers include peptide neurotoxins, mainly ω-conotoxins and ω-agatoxins (Olivera *et al.*, 1994; Tareilus and Breer, 1995).

On the other hand, as already mentioned, the entry of Ca^{2+} through the NMDA-receptor-associated channels seems to be more directly related to neuronal damage than that occurring through VSCC. Evidence for this includes the demonstration that exposure to glutamate or to NMDA-receptor agonists results in neuronal death, and that this effect is prevented by NMDA receptor antagonists (Choi, 1988; Hartley *et al.*, 1993; Tymianski *et al.*, 1993). However, it should be emphasized that this is not the only mechanism of cell death. Other important factors include oxidative stress leading to free radical formation, deficiencies in the scavenger or metabolic mechanisms that normally dispose of free radicals, derangement of cytoskeletal proteins, or mitochondrial functional damage resulting in the disruption of oxidative phosphorylation (Coyle and Puttfarcken, 1993; Olanow, 1993; Martin *et al.*, 1994).

In addition to the above-mentioned compounds that are capable of blocking Ca^{2+} channels and neurotransmitter release, or of inducing neurodegeneration, the inorganic dye ruthenium red (RuR) has been shown to possess both effects, depending on the experimental conditions used. In contrast to the simple ruthenium salts such as $RuCl_3$, RuR (ruthenium oxychloride ammoniated) is a complex polycationic compound of relatively high molecular weight. Although some variants of its formula have been proposed, the following is generally accepted:

$$[(NH_3)_5Ru–O–Ru(NH_3)_4–O–Ru(NH_3)_5]^{6+}6Cl^-.$$
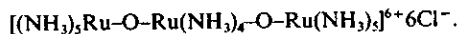
The effects of RuR on the nervous system have been extensively studied in our laboratory for a number of years. Several *in vitro* and *in vivo* experimental approaches have been followed, including RuR actions on Ca^{2+} influx and neurotransmitter release in brain slices and isolated nerve endings (synaptosomes), on motor behavior in rats, mice and cats, and induction of neuronal damage after intracerebral microinjections and in neuronal cultures. The results of these investigations, together with data from other laboratories, indicate that RuR is an important and useful tool for studying the relationships between calcium channels and neurotransmitter release, as well as the mechanisms of neuronal death. Furthermore, the injection of RuR in discrete brain regions may provide useful information on the function of neural pathways within the brain. The purpose of this article is to review the main findings on these lines of investigation.

RuR BINDING AND EFFECTS ON Ca^{2+} INFLUX AND NEUROTRANSMITTER RELEASE

Because of its polycationic nature, RuR is able to bind to negative charges on the external membrane surface. Since RuR is also electron-dense, it has been used as a membrane stain in electron microscopy (Luft, 1971a,b). In this type of study, the compound

is added to the fixative solutions, so that the tissue to be examined is fixed simultaneously to the staining. On the other hand, until recently relatively few studies have been carried out on the effects of RuR in nervous tissue when added in physiological solutions.

Using biochemical techniques, we have analysed the binding of RuR to freshly isolated brain synaptosomes, in comparison to the well known VSCC blocker La^{3+}, as well as the interaction between these two cations (Tapia et al., 1985a). The results of these experiments showed that both RuR and La^{3+} bind very rapidly to the synaptosomal membrane. In a Na^+- and Ca^{2+}-containing medium, one binding site for RuR was identified kinetically, with a K_d of $3.7\,\mu M$, and two binding sites for La^{3+} ($K_d = 2.3\,\mu M$ for the high affinity site and 63 μM for the low affinity site). Furthermore, RuR competitively blocked the binding of La^{3+}, suggesting that the high affinity site for this cation is shared by RuR. This conclusion was supported by the fact that La^{3+} completely inhibited the binding of RuR to the synaptosomal membrane, although only weakly displaced it when added to synaptosomes previously incubated with RuR (Tapia et al., 1985a). As summarized in Table 1, RuR also blocks the binding of Ca^{2+} and of Tb^{3+} to synaptosomes, as well as that of the N-type VSCC blocker ω-conotoxin, whereas the binding of dihydropyridines, L-type blockers, is not affected.

The above findings clearly suggest that RuR interacts with Ca^{2+} sites located in the nerve ending membrane. In order to test whether such sites are related to VSCC, and whether the influx of Ca^{2+} through these channels is in turn involved in the mechanisms of neurotransmitter release, the effect of RuR on these two parameters was studied in synaptosomes. The results of these experiments show that RuR at micromolar concentrations close to its K_d binding value inhibits the influx of Ca^{2+} induced by K^+-depolarization (Tapia, 1985; Tapia et al., 1985b; Arias and Tapia, 1986; Hamilton and Lundy, 1995). This inhibitory effect has been confirmed by measuring fluorimetrically the K^+-induced increase in intrasynaptosomal Ca^{2+} concentration, without any significant modification of membrane polarity (Taipale et al., 1989).

The blockade of Ca^{2+} influx by RuR was correlated with an inhibition of the Ca^{2+}-dependent release of neurotransmitters, including γ-aminobutyric acid (GABA), glutamate, acetylcholine (ACh) and dopamine (Tapia and Meza-Ruiz, 1977; Tapia et al., 1985a; Hamilton and Lundy, 1995), when synaptosomes were depolarized by high K^+ concentrations. RuR also blocked the release induced by 4-aminopyridine, a drug that stimulates the spontaneous transmitter release in a strictly Ca^{2+}-dependent manner (Tapia and Sitges, 1982; Tapia et al., 1985b). These effects of RuR on Ca^{2+} entry and neurotransmitter release are generally similar to those produced by La^{3+} (Tapia et al., 1985b).

The action of RuR on the Ca^{2+}-dependent release of neurotransmitters has been also found in other in vitro preparations, such as frog neuromuscular junction (Person and Kuhn, 1979), rat nerve-diaphragm preparation (Hamilton and Lundy, 1995) and hippocampal slices (Wierasko, 1986). In addition, largely based on the above studies from our laboratory, RuR has been established as an important blocker of the

Table 1. Described effects of RuR on Ca^{2+} membrane sites and transmitter release in nervous tissue preparations

Preparation	Binding of Ca^{2+} and VSCC blockers	Ca^{2+} influx	Transmitter release inhibition
		Stimulated by K^+-depolarization	
Synaptosomes (rat, mouse, chicken, insect)	Blocks binding of Ca^{2+}, La^{3+}, Tb^{3+}, ω-conotoxin, no effect on DHP	Inhibits	GABA, glutamate, dopamine, acetylcholine
		Stimulated by capsaicin	
Sensory fibers and neurons	—	Inhibits	Peptides
		Induced by nerve stimulation	
Nerve-muscle (rat, frog)	—	—	Acetylcholine

DHP, dihydropyridines. References for synaptosomes: Madeira and Antunes-Madeira (1973); Kamino et al. (1976); Tapia and Meza-Ruiz (1977); Breer and Jeserich (1981); Tapia (1985); Tapia et al. (1985a); Arias and Tapia (1986); Massieu and Tapia (1988); Taipale et al. (1989); Hamilton and Lundy (1995). References for sensory neurons: Maggi et al. (1988); Maggi et al. (1989); Wood et al. (1988); Dray et al. (1990); Holzer (1991). References for nerve-muscle: Person and Kuhn (1979); Hamilton and Lundy (1995).

Ca^{2+} influx and the Ca^{2+}-dependent release of pep-
tides induced by capsaicin in several nervous tissue
preparations (Chahl, 1989; Amann *et al.*, 1989; Maggi
et al., 1988, 1989; Wood *et al.*, 1988; Dray *et al.*, 1990;
Amann and Maggi, 1991; Holzer, 1991). A summary
of the actions of RuR on Ca^{2+} sites and transmitter
release is shown in Table 1.

The work *in vitro* reviewed up to this point dem-
onstrates that, as in the case of the inhibitory effect
of La^{3+}, both in central synapses and in peripheral
junctions the effects of RuR are due to the blockade
of VSCC located in the nerve ending membrane. One
important aspect, however, is the type of VSCC affec-
ted by RuR. This question has been addressed by
studying the effect of RuR on the binding to syn-
aptosomal membranes of antagonists of different
types of VSCC in synaptosomes (Massieu and Tapia,
1988; Hamilton and Lundy, 1995). The results show
that the binding of dihydropyridines like nitrendipine
or PN200-110, which are L-type channel antagonists,
is not affected by RuR, indicating that this type of
channel does not participate in any important way in
the entrance of Ca^{2+} linked to transmitter release. In
agreement with this conclusion, neither nitrendipine
nor nifedipine inhibited the Ca^{2+}-dependent trans-
mitter release induced by K^+-depolarization or by 4-
aminopyridine and, furthermore, the L-channel agon-
ist Bay K8644 failed to affect both the spontaneous
and the K^+-stimulated release (Massieu and Tapia,
1988). On these bases, it was postulated that RuR
interacts with the *N*-type VSCC, a conclusion strongly
supported by similar recent work in synaptosomes
and in rat nerve–diaphragm preparation, using dihy-
dropyridines, ω-conotoxins and ω-agatoxins as antag-
onists (Hamilton and Lundy, 1995). In this study it
was shown that the effects of RuR are mediated
mainly by its interaction with the *N*-type VSCC,
although the *P*-type seems to be involved also. Thus,
these two types of VSCC seem to be specifically related
to the coupling between Ca^{2+} entry and neuro-
transmitter release.

It has been mentioned that RuR is able to bind to
the negative charges of the membrane surface. Such
negative charges are mainly those of the sialic acid
residues of gangliosides and glycoproteins, which are
abundant on the external surface of neuronal mem-
branes. In this regard, it has been shown in hip-
pocampal slices that the removal of sialic acid by
treatment with neuraminidase, or the addition of
exogenous gangliosides, notably delays the blockade
of synaptic transmission by RuR, indicating a link
between sialic acid, RuR and Ca^{2+} channels (Wier-
asko, 1986). Such a link was previously suggested in

experiments in synaptosomes showing that when the
screening of negative surface charges normally exerted
by endogenous Ca^{2+} is disrupted by chelators of this
cation, the membrane becomes more permeable to
Na^+ and this change in membrane permeability is
blocked by RuR (Arias *et al.*, 1984).

RuR AND La^{3+} EFFECTS AND INTERACTIONS *IN VIVO*

A series of experiments carried out *in vivo* strongly
suggests that the described inhibitory actions of RuR
and La^{3+} on Ca^{2+} influx and on the Ca^{2+} dependent
release of neurotransmitters occur also in the living
animal and result in severe motor alterations.

When RuR was administered intraperitoneally
(i.p.) to mice, rats or cats, a notable flaccid paralysis
occurred after a few minutes and lasted for 3–6 h. This
paralyzing action can be ascribed to an inhibition of
the Ca^{2+}-dependent release of acetylcholine at neuro-
muscular junctions, because of the following findings.
(a) In the mouse, the RuR-induced paralysis was mim-
icked by the i.p. administration of the calcium chelator
EDTA, and the paralysis induced by both compounds
was reversed by the elevation of Ca^{2+} levels produced
by $CaCl_2$ administration (Tapia *et al.*, 1976). In the
rat, the paralyzing effect lasted for about 3 h and was
followed by convulsive seizures (García-Ugalde and
Tapia, 1991). (b) In the cat, the diminution of mus-
cular tone was antagonized by the administration of
the cholinergic agonist carbachol, indicating that the
response of the ACh receptor located in the muscle
postsynaptic membrane was not affected (Tapia *et al.*,
1976). (c) The RuR-induced paralysis was completely
antagonized by the systemic administration of drugs
that induce the release of neurotransmitters in a Ca^{2+}-
dependent manner, such as guanidine and the afore-
mentioned 4-aminopyridine. It must be emphasized
that the latter two drugs were very effective when
administered to mice several minutes after RuR, when
the animals showed complete paralysis (Tapia, 1982,
1985). (d) In contrast to guanidine and 4-amin-
opyridine, the administration of La^{3+} to mice par-
alyzed by previous RuR injection did not reverse the
paralysis. However, when La^{3+} was administered
before RuR, it completely prevented the occurrence
of flaccid paralysis (Tapia, 1985). This finding can be
correlated with the binding studies described above,
since La^{3+} was able to inhibit the binding of RuR to
nerve endings membrane but only weakly displaced
the previously bound RuR.

It is worth mentioning that the paralyzing action of
the i.p. administration of RuR closely mimicks the
motor alterations observed in Lambert–Eaton myas-

thenic syndrome in humans. In this autoimmune disease, antibodies are formed against VSCC, resulting in a blockade of Ca^{2+} entry and ACh release, which in turn produces muscular weakness and paralysis. A recent work in nerve–diaphragm preparations from mice treated with serum obtained from Lambert–Eaton patients has shown that the antibodies bind to the N-type VSCC and not to the L-type channels of the motor nerve endings (Smith et al., 1995). Since this process and its consequences are similar to the action of RuR described above, we suggest that the systemic treatment with RuR may be a good experimental model of Lambert–Eaton myasthenic syndrome.

Another related aspect needs to be revised. According to the binding and neurotransmitter release studies in vitro, which indicate that RuR and La^{3+} share a presynaptic membranal site and possess similar effects, it should be expected that the systemic administration of La^{3+} itself would also result in flaccid paralysis. However, when we injected $LaCl_3$ alone, at the doses which, as described above, antagonized the paralyzing action of RuR, no paralysis was observed (Tapia, 1982, 1985). In view of the clear antagonist effect, it cannot be argued that the injected La^{3+} is chelated or somehow eliminated before reaching the muscles, and therefore a different explanation must be sought for the lack of paralyzing effect of La^{3+}. A plausible interpretation is based on the fact that this cation exerts a dual action at neuromuscular junctions: it markedly increases the spontaneous release of ACh and it inhibits the depolarization-stimulated release of this transmitter (Heuser and Miledi, 1971; Miledi et al., 1980). Thus, it can be concluded that, in vivo, the former action of La^{3+} predominates over the latter and that it is more important for maintaining a normal muscular tone. The fact that, contrary to La^{3+}, RuR inhibits the spontaneous release of ACh in neuromuscular junctions (Person and Kuhn, 1979) is in agreement with this interpretation.

In other experiments, we have studied the effects of RuR and La^{3+} when injected intracranially. The dye has been administered into the cisternae of unanesthetized mice, as well as into the cerebral ventricles of rats and cats, using stereotaxic techniques. In the three species this treatment resulted in the appearance of intense generalized convulsions, which in the cat lasted for over 24 h and were accompanied by continuous electroencephalographic discharges characteristic of status epilepticus (Tapia et al., 1976; Belmar et al., 1995). We have also found that the K^+-depolarization-induced release of GABA was inhibited by more than 50% in synaptosomes isolated from the brain of mice killed during convulsions produced by RuR (Meza-Ruiz and Tapia, 1978).

The intracisternal injection of $LaCl_3$ to mice also produced convulsions, which, together with the previous findings, suggests that both La^{3+} and RuR inhibit transmitter release in central synapses when injected in the cerebrospinal fluid, and that epileptic seizures occur as a consequence of such inhibition (Tapia, 1985). However, subsequent investigations on the action of RuR when administered directly in brain parenchyma, into small, discrete regions, indicate that under these conditions RuR is able to penetrate the neurons and behaves as a potent neurotoxin. These experiments will be reviewed in the following section.

HYPEREXCITATION AND RuR NEUROTOXICITY

The effects of RuR described in the previous section clearly point to a rapid action of RuR on neuromuscular and interneuronal communication, owing to the binding of the drug to the presynaptic plasma membrane. Moreover, the experiments in vitro showed no indication of the presence or action of RuR inside the cells. Therefore, it was highly surprising to find that when RuR was stereotaxically microinjected in rat brain and the injected region was examined histologically, the dye was clearly located within neuronal somata, whereas neither the neuropil nor glial cells was stained. This has been found both in neurons of the substantia nigra reticulata (SNR) (Tapia and Flores-Hernández, 1990) and in the pyramidal cells of the CA1 area of the hippocampus (García-Ugalde and Tapia, 1991; Belmar et al., 1995). Furthermore, after the injection of RuR in the lateral ventricle the dye was also located inside the neuronal somas surrounding the ventricle (Belmar et al., 1995).

The behavioral effects of the intracerebral administration of RuR were remarkable, and their characteristics were dependent on the region injected. When the dye was injected unilaterally into the SNR it produced long lasting (up to 5 days) motor alterations, characterized by contraversive intense circling movements and head orientation (Tapia and Flores-Hernández, 1990). The most prominent behavioral modification after its intrahippocampal (i.h.) injection was the appearance of a well defined motor abnormality known as 'wet-dog shakes', accompanied by a series of symptoms that have been described as limbic type seizures, such as grooming, rearing with forelimb clonus, masticatory movements and head nodding (García-Ugalde and Tapia, 1991; Belmar et al., 1995). Neither of these effects could be ascribed to a direct RuR-induced inhibition of neurotransmitter release

because, differently from the intracisternal injection described above, when the basal and the K^+-stimulated release of GABA was studied in synaptosomes or in slices of the injected SNR and hippocampus, no differences were observed in comparison with the contralateral non-injected tissue (Tapia and Flores-Hernández, 1990; García-Ugalde and Tapia, 1991). Furthermore, in the case of the CA1 area, neither the coinjection of the GABA$_A$ receptor agonist 4,5,6,7-tetrahydroisoxazol[5,4-c]pyridin-3-ol (THIP), nor that of the GABA uptake inhibitor nipecotic acid, diminished the frequency of wet-dog shakes produced by RuR (García-Ugalde and Tapia, 1991).

These observations led us to postulate that the mechanism of action of RuR *in vivo* was not the same when administered systemically as when injected into cerebral tissue. In the latter case there seems to be a very effective mechanism for the internalization of RuR into neuronal bodies, sparing the neuropil and glial cells. Since all motor abnormalities produced by the dye indicate the induction of notable neuronal hyperexcitability, and those observed after injection in the hippocampal CA1 area are similar to those produced by the potent excitatory amino acid receptor agonist kainic acid (Ben-Ari *et al.*, 1981; Lothman and Collins, 1981), we have concluded that the intraneuronal RuR is responsible for the hyperexcitability.

A clue to the mechanism for this hyperexcitability may be the fact that the RuR-containing neurons are rapidly damaged. In fact, we have observed, both by light and electron microscopic examination of the RuR-injected CA1 region, that neurons containing the dye are destroyed: they look shrunken, with loss of cytoplasmic organization, remarkable vacuolization and chromatin disaggregation. These changes occurred within 1 h of RuR injection, and after 5-9 days they were reflected in neuronal loss and complete disruption of the CA1 cell layer architecture (Belmar *et al.*, 1995). Based on all these findings, we propose that, when administered directly into the cerebral parenchyma, RuR is rapidly taken up by neuronal somas and once inside produces hyperexcitation and it is extremely neurotoxic. This might represent a kind of excitotoxicity, a term usually referred to the toxicity caused by overactivity of excitatory amino acid receptors.

The results obtained so far leave open the following questions: What neural circuits are involved in the motor alterations produced by RuR? What is the mechanism of RuR entry into the neurons and for its apparent selectivity for neuronal bodies? What is the mechanism of the hyperexcitation and neurotoxicity? In the next sections some experiments aimed at solving these questions will be described.

EXCITATORY AMINO ACIDS AND RuR-INDUCED HYPEREXCITABILITY

Synaptic neurotransmission mediated by glutamate, the most important excitatory amino acid, seems to play a major role in the mechanisms of seizures, particularly through the NMDA receptor. In fact, glutamate receptor agonists are potent convulsant agents and, conversely, NMDA receptor antagonists protect against convulsions in a variety of models of experimental epilepsy (Löscher *et al.*, 1988; Dingledine *et al.*, 1990; Chapman, 1991). Therefore, it was reasonable to postulate that, if RuR is producing neuronal hyperexcitation, excessive glutamatergic neurotransmission mediated by NMDA receptors could be involved. In order to test this hypothesis, we have recently studied the effect of some antagonists of the NMDA and non-NMDA receptors on the convulsant action of the intracerebroventricular (i.c.v.) and i.h. administration of RuR. For comparison, we also tested the possible protection by antiepileptic GABAergic compounds and by diphenylhydantoin.

As summarized in Table 2, partial protection against the effects of RuR was observed with several NMDA receptor antagonists and with the GABAergic compounds, aminooxyacetic acid and valproic acid, when administered i.c.v. or i.p., but not when

Table 2. Protection by NMDA receptor antagonists and GABAergic compounds against convulsions and wet-dog shakes produced by the intracerebroventricular (i.c.v.) and intrahippocampal (i.h.) administration of RuR in rats

i.c.v. RuR (2.1 nmol)	Mean number of: Tonic convulsions/h ± SEM (% of rats with status epilepticus for > 20 min)
No antagonist	8.1 ± 1 (31%)
CGP-37849 (i.p., 10 mg/kg))	5.7 ± 2.7 (0%)
Aminooxyacetate (i.p., 50 mg/kg)	3.1 ± 1 (0%)
Valproate (i.p., 300 mg/kg)	1.6 ± 0.9 (0%)
i.h. RuR (1 nmol)	Wet-dog shakes/2 h ± SEM
No antagonist	86 ± 5.4
CPP (i.c.v., 2 nmol)	11 ± 3.5
(i.h. (0.4 nmol)	127 ± 18
CGP-37849 (i.p., 10 mg/kg)	19 ± 5.5
(i.h. (0.4 nmol)	94 ± 14
MK-801 (i.p., 1 mg/kg)	31 ± 4.5
Aminooxyacetate (i.p. 50 mg/kg)	13 ± 4.5
Valproate (i.p. 300 mg/kg)	49 ± 15

Data from Belmar *et al.* (1995). CGP-37849, d,l-[E]-2-amino-4-methyl-5-phosphono-3-pentanoic acid; CPP, (±)3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; MK-801, (+)5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine hydrogen maleate. Drugs were injected 30 min before RuR (except when coinjected i.h.) at the doses and routes of administration indicated. Neither i.c.v. CPP nor i.p. MK-801 was effective against i.c.v. RuR.

co-injected i.h. with RuR. The non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione was ineffective. These findings indicate that NMDA receptors play a significant role in the hyperexcitation induced by RuR, and that such receptors are located in cerebral regions different from the injected area (Belmar *et al.*, 1995). Such participation of remote brain areas in the behavioral alterations produced by the RuR-induced stimulation of the hippocampus has been previously shown using the serotonin 5-HT$_2$ receptor antagonist ketanserin, which was very effective in preventing the occurrence of wet-dog shakes when administered i.p. but not when co-injected i.h. with RuR (Garcia-Ugalde and Tapia, 1991). From these studies it can be concluded that the behavioral alterations induced by i.n. RuR are mediated by the activation of neuronal circuits involving NMDA and serotonin receptors. Since aminooxyacetic and valproic acids are believed to facilitate the general GABAergic inhibitory tone in the brain, their protective action against RuR hyperexcitability is not inconsistent with the above interpretation.

MECHANISMS OF RuR NEUROTOXICITY: STUDIES IN CELL CULTURES

As described in the introductory section, an increase of cytoplasmic Ca^{2+}, as occurring after overactivation of NMDA receptors, has been causally related to neuronal death. Since RuR has been located inside the neurons shortly after its intracerebral administration and the resulting neuronal damage is similar to that described after treatment with NMDA agonists, it is possible that RuR may bind to intracellular organelles or proteins responsible for Ca^{2+} buffering, and thus prevent the trapping of the divalent cation and consequently increase its cytoplasmic concentration. In fact, it has been described that RuR can bind to the Ca^{2+}-binding site of several Ca^{2+} sequestering proteins, such as calmodulin and calsequestrin (Sasaki *et al.*, 1992; Charuk *et al.*, 1990) and, when injected into cultured neurons, notably diminishes the intracellular buffering capacity after drug-induced elevation of the cytoplasmic Ca^{2+} (Thayer and Miller, 1990; Marrion and Adams, 1992). Furthermore, RuR inhibits Ca^{2+} transport in isolated mitochondria (Moore, 1971; Reed and Bygrave, 1974) and prevents brain tubulin polymerization and induces the disassembly of microtubules, although this effect cannot be reversed by Ca^{2+} (Deinum *et al.*, 1981). We have recently studied the possibility of some of these mechanisms using primary neuronal and glial cultures. Initially the aim of these experiments was to test whether RuR was

capable of inducing cell damage in cultures and whether this damage was specific for neurons, as suggested by the results of the intracerebral administration of RuR *in vivo*.

In this study cell damage was assessed by the reduction of a tetrazolium salt, which is a reaction dependent on the state of the mitochondrial respiratory chain. As shown in Fig. 1, after relatively long incubation periods with micromolar RuR con-
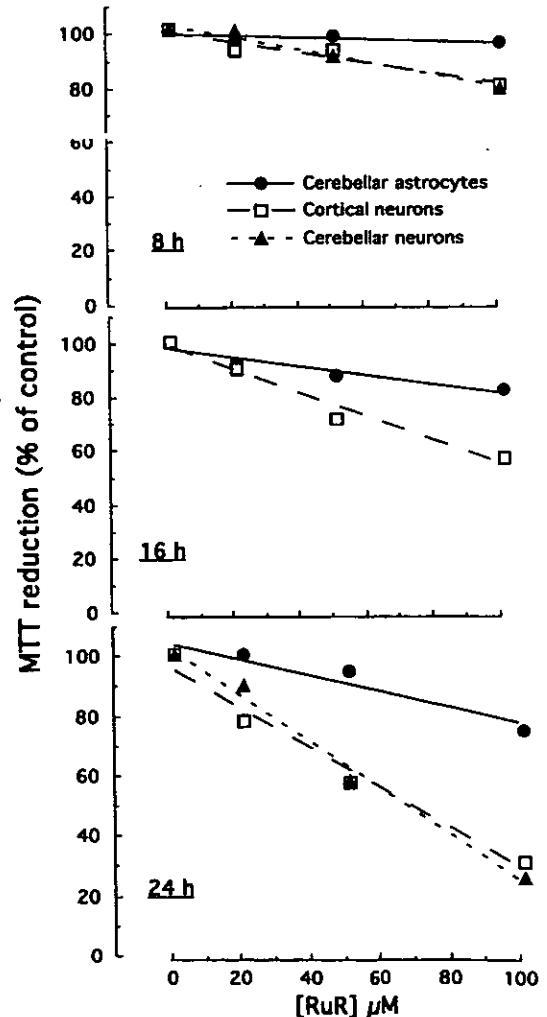

Fig. 1. RuR-induced neurodegeneration as assessed by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Neuronal and glial cultures were incubated with different RuR concentrations during the time periods indicated on each panel. Data from Velasco *et al.* (1995).

centrations, a notable dose-dependent inhibition of this function was observed in neuronal cultures, but not in glial cultures, thus confirming the specificity of the cell damage and suggesting that derangement of mitochondrial oxidative function might be the primary mechanism of RuR-induced cell damage. However, it was also found by immunocytochemical studies of the neuronal cultures that RuR produced notable alterations in the α-tubulin immunoreactivity in the somata, as well as in the neural processes, which looked detached and fragmented (Velasco *et al.*, 1995). With the available information it is not possible to know whether these changes were due to RuR binding to tubulin or were secondary to other changes, such as alteration in intracellular Ca^{2+} homeostasis.

Neuronal damage was also observed in the cultures by light microscopy. Although not all cells were affec-

ted, both RuR-treated cortical and cerebellar neurons showed remarkable alterations, although, differently from the experiments *in vivo* and in parallel with the tetrazolium reduction, this effect required long incubation times (8–24 h). The damage was characterized by cell vacuolization and loss and fragmentation of neurites and, in agreement with the observations *in vivo*, pure astrocyte cultures were not affected (Velasco *et al.*, 1995). From these experiments it was concluded that the RuR cell selectivity was due to a differential permeability, since the microscopic observations showed that the dye was present inside the neurons but not in the glial cells. The mechanism of the selective RuR entrance into neurons is still unknown.

We have recently examined the RuR-treated neuronal cultures by scanning electron microscopy. As exemplified in Figs 2 and 3, this technique revealed
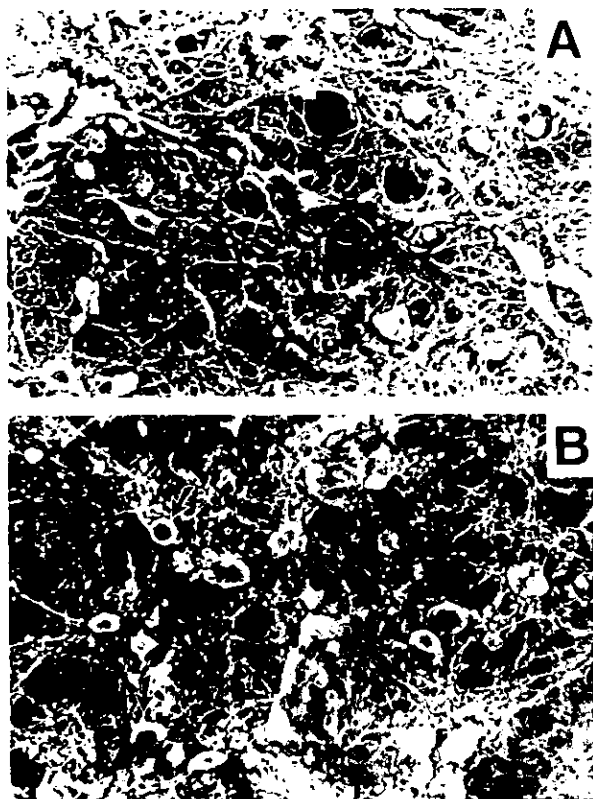

Fig. 2. Scanning electron micrograph of cortical neurons cultured for 4 days. Cultures were washed with phosphate buffer–saline pH 7.4, fixed in 3% glutaraldehyde, post-fixed in buffered 2% osmium tetroxide, dehydrated in a graded series of ethanol and dried to critical-point using liquid CO_2. Samples were mounted on copper holders, coated with gold and observed in a Jeol 5410LV scanning electron microscope: (A) control; (B) after 16 h incubation with 100 μM RuR. Note the disruption of neural processes and the spherical appearance of several neurons in the RuR-treated cultures (arrows). Bar = 10 μm.
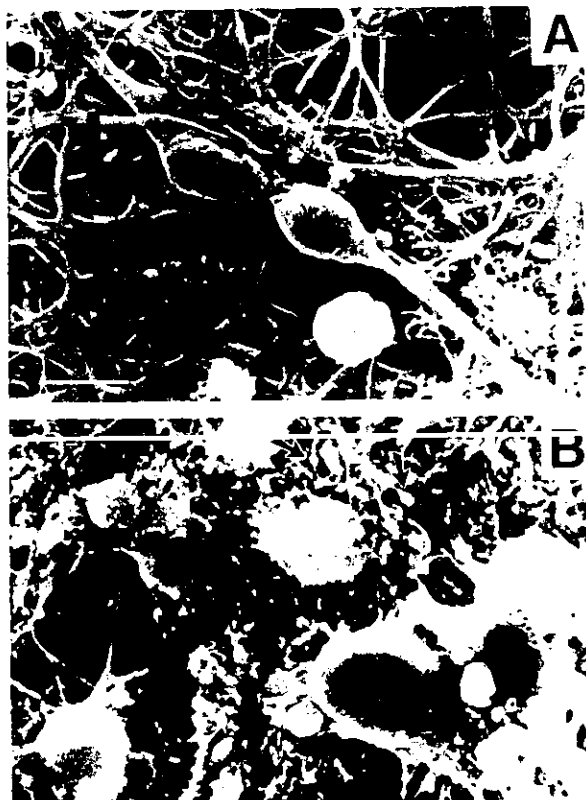
Fig. 3. Higher magnification of different fields of the cultures shown in Fig. 2: (A) control; (B) RuR-treated. Note the fragmentation and the varicosities (arrows) of neurites, and the alterations in the shape and surface of the neurons, in comparison with the control. Bar = 5 μm.

interesting details of the loss and fragmentation of neurites, as well as changes in the shape of neuronal somata, which became more spherical, and alterations in the membrane surface, that appeared very rough. Confirming the previous light microscopy observations, there seems to be a population of neurons not affected by RuR.

CONCLUDING REMARKS

The experimental data reviewed in this article demonstrate that RuR exerts several interesting effects on neuronal function and provides a novel approach for studying the mechanisms of neuronal death and the functional relations between neural circuits in the CNS. More specifically, RuR seems to be a useful tool for exploring the following physiological and pathological processes, both *in vitro* and *in vivo*:

1. Calcium movements through specific VSCC in the neuronal membrane, especially the presynaptic membrane, and their consequences on the Ca^{2+}-dependent neurotransmitter release. In particular, the effects provide relevant information on the relationship between the influx of Ca^{2+} through specific VSCC and the intraterminal Ca^{2+} pool involved in the triggering of transmitter release. Furthermore, RuR might also shed light on the mechanisms of release of peptides or hormones, as has been already shown in the case of the capsaicin-induced peptide release.

2. The mechanisms of neuronal cell death, including the role of intracellular Ca^{2+}, cytoskeletal proteins and mitochondrial oxidative function. These aspects, which have been the subject of numerous studies *in vitro*, especially in tissue cultures, demand new approaches that permit a better understanding

of the intracellular mechanisms of brain damage in the living animal, and the data obtained so far suggest that RuR may be a relevant tool in this direction.

3. The alteration of neural circuits as related to the motor behavior of the animals. This approach can be followed by damaging small populations of neuronal bodies in selected brain nuclei, by means of the microinjection of RuR, and studying the role of the efferent pathways in a particular behavioral function. It has been already shown that this application of RuR may be important in the case of several disturbances of motor functions, such as epilepsy, but it is obvious that many functions of the brain could be studied, depending on the brain region injected.

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OBJETIVOS

Como se mencionó en la introducción, el RR es un compuesto muy interesante porque es capaz de producir muerte neuronal sin afección de otros tipos celulares al inyectarse en el parénquima cerebral (artículo 1). No es claro cuáles son las acciones tóxicas del RR sobre las neuronas, por lo que a lo largo de esta tesis se estudiaron varias posibilidades usando como modelo principalmente a las neuronas en cultivos primarios. Así, los objetivos fueron:

1) Confirmar en cultivos primarios que el RR es una neurotoxina que no afecta a otras células presentes en el SNC (artículo 2).

2) Realizar observaciones ultraestructurales de los efectos del RR sobre las neuronas cultivadas (artículo 4).

3) Establecer si el RR debe ser internalizado a las neuronas para inducir muerte y qué sitios de la membrana podrían estar participando en su unión/entrada (artículos 3 y 5).

4) Probar si la homeostasis de Ca^{2+} intracelular participa en la neurodegeneración inducida por RR (artículo 4).

5) Establecer la participación del bloqueo de los canales de Ca^{2+} por RR en la toxicidad (artículo 4).

6) Investigar si la activación de los receptores a Glu contribuye a la neurotoxicidad (artículo 4).

7) Establecer si el RR induce apoptosis en neuronas (artículo 4).

8) Probar si el RR puede interferir con la función mitocondrial usando

mitocondrias aisladas de cerebro (artículo 4).

9) Investigar si proteínas neurona-específicas son las responsables de la entrada del RR a las células y de la muerte al expresar el mRNA extraído de las neuronas cultivadas en ovocitos de Xenopus (artículo 5).

Selective Neurotoxicity of Ruthenium Red in Primary Cultures*

Iván Velasco,[1] Julio Morán,[1] and Ricardo Tapia[1,2]

The inorganic dye ruthenium red (RuR) has been shown to be neurotoxic in vivo when injected intracerebrally. In this work the toxicity of RuR was compared in primary cultures of rat cortical neurons, cerebellar granule neurons and cerebellar astroglia. Microscopic examination of the cultures revealed that RuR penetrates the somata of both types of neurons used and produces vacuolization and loss and fragmentation of neurites. In contrast, no RuR was seen inside cultured astrocytes and no morphological signs of damage were observed in these cells. RuR toxicity was also assessed by immunocytochemistry of α-tubulin and by biochemical measurement of the reduction of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by the cultured cells. The morphological alterations in the neurons were closely correlated with loss of tubulin immunoreactivity and particularly with a notable decrement in the ability to reduce MTT. Using the latter parameter, it was found that neuronal damage was independent of the age of the cultures, augmented progressively with time of incubation with RuR, from 8 to 24 h, and showed a clear dose-response curve from 20 to 100 μM RuR. Astrocytes showed only a slight decrease in MTT reduction after 24 h of incubation with 100 μM RuR. It is concluded that RuR seems to be toxic for neurons but not for astroglia, and that this selectivity is probably related to the ability of the neurons to internalize the dye. The possible mechanisms of RuR penetration and neuronal damage are discussed.

KEY WORDS: Ruthenium red; neuronal cultures; neurotoxicity; astrocytes.

INTRODUCTION

Ruthenium red (RuR) is an inorganic dye possessing several effects on calcium-related functions in biological preparations, such as inhibition of Ca^{2+} uptake in mitochondria (1-3), block of the caffeine-induced release of Ca^{2+} in sarcoplasmic reticulum (4) and inhibition of Ca^{2+}-ATPase activity (2,5). In synaptosomes, RuR binds to membranal calcium-related sites (6), inhibits Ca^{2+} binding in a non-competitive way (7) and blocks the entry of Ca^{2+} induced by K^+-depolarization

(6,8). In the neuromuscular junction, RuR inhibits spontaneous as well as calcium ionophore-induced transmitter release (9,10).

RuR is also able to interact with cytoskeletal proteins. It prevents polymerization of brain tubulin and induces the disassembly of microtubules, although as this effect cannot be reversed by Ca^{2+}, RuR probably binds to a site different from that of the cation (11). Several Ca^{2+}-binding proteins, such as calmodulin and calsequestrin, are also capable of binding RuR. In both proteins the binding site of RuR seems to be related to that of Ca^{2+}, as there is a reciprocal antagonism of binding (12,13).

In cultured ganglion neurons, intracellular injection of RuR potentiates the caffeine-induced release of Ca^{2+} from intracellular stores and slows down the buffering

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of the resulting elevated cytoplasmic Ca^{2+} levels (14). Similarly, in dorsal root ganglion cells, RuR inhibits the largest component of Ca^{2+} buffering (15).

Experiments in vivo on the effects of stereotaxic microinjection of RuR into the rat substantia nigra reticulata (16), the CA1 region of the hippocampus (17) or the lateral cerebral ventricle (E. Belmar and R. Tapia, in preparation) suggest a predominant affinity of the dye for neurons. Histological examination of the three microinjected regions has shown that RuR is preferentially located inside neuronal somata. This neuronal penetration was associated with the induction of motor alterations that were long-lasting and distinct, namely contralateral turning behavior, limbic seizures and wet-dog shakes, and intense generalized convulsions, after intranigral, intrahippocampal or intraventricular administration, respectively. From these studies we have proposed that when it is administered intracerebrally RuR produces a marked neuronal hyperexcitation eventually leading to cell death, which implies that RuR acts as an excitotoxic compound. In support of this hypothesis, light microscopy examination of the injected hippocampus has revealed a notable disruption of the CA1 cellular architecture, and in addition electron microscopy observations showed intense vacuolization in the RuR-containing neuronal somata (18).

The aim of the present work was to test whether the apparent neuronal specificity of RuR observed in brain tissue in vivo was also demonstrable in primary neuronal cultures, as an approach to investigating the mechanism of action of the dye. For this purpose the effect of RuR was compared in cultured cortical and cerebellar neurons and in cultured cerebellar astrocytes.

EXPERIMENTAL PROCEDURE

Materials. Fetal calf serum and penicillin/streptomycin were obtained from GIBCO (Gaithersburg, MD, USA). RuR, poly-L-lysine (mol. wt. > 300,000), trypsin, soybean trypsin inhibitor, DNAse I, cytosine arabinoside, diaminobenzidine, and antibodies against α-tubulin were purchased from Sigma (St. Louis MO, USA). The batches of RuR used were checked by obtaining the absorption spectra and the maximum at 534 nm was used as criterion for calculating the concentration of the dye. As previously reported (19), no important spectrophotometric or toxicity differences were seen between different batches. Antibodies to murine IgG were obtained from Vector (Burlingame, CA, USA). All other chemicals were of the purest grade available from regular commercial sources.

Cell Cultures. Primary cultures of cortical neurons were prepared by seeding cell suspensions obtained from forebrains of 17 day-old fetuses from pregnant Wistar rats bred on the premises under standard animal house conditions, as previously described (20). For cerebellar granule neurons and cerebellar astroglial cells tissue was from 8 day-old rats, as described by Morán and Patel (21) and Morán and Rivera-

Gaxiola (22). Briefly, for neuronal cultures the dissociated cell suspensions from the cortex or cerebellum were plated at a density of $260-310 \times 10^3$ cells/cm² (100×10^3 cells/cm² for the immunocytochemical experiments) in plastic 35 mm-diameter Petri dishes or multiwell clusters previously coated with poly-L-lysine (5 μg/ml), containing basal Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 25 mM KCl, 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. The culture dishes were incubated at 37°C in a humidified 5% CO_2, 95% air atmosphere. Cytosine arabinoside (10 μM) was added at about 20 h after seeding. For cerebellar astrocytes the cells were seeded at a lower density (130×10^3 cells/cm²), with 5 mM KCl and in the absence of poly-L-lysine and cytosine arabinoside. Under these conditions the proportion of neurons in the neuronal cultures is 95%-97%, and the astroglial cultures contained more than 95% positive cells for glial fibrillary acidic protein (22).

Neuronal cultures were maintained in the same medium for 4-6 days in vitro (DIV) without change of medium, or for 14 DIV. In the latter case, an excess of culture medium was present (1 ml instead of 0.5 ml), cytosine arabinoside was added 4 days after platting, and 3.75 mM glucose was added at 11 days. Glial cell cultures were kept at least 2 weeks in vitro, with change of medium every 3 days. After these maintenance periods, the effect of RuR was tested by substituting the medium for a freshly prepared RuR-containing Locke medium of the following composition (in mM): NaCl 154, KCl 5.6, NaHCO₃ 3.6, CaCl₂ 2.3, MgCl₂ 1.2, glucose 5.6, HEPES 5 (23). RuR was tested at the concentrations and time periods indicated under Results. Control cultures treated with Locke medium without RuR were included in each experiment and were handled in parallel. All experiments were performed in duplicate using cultures prepared from the same set of animals. Cell morphology and appearance of the cultures were monitored by phase contrast microscopy in each experiment before RuR treatment. Poorly preserved cultures were discarded.

Assessment of Cell Damage. The following approaches were followed for assessment of the damage produced by RuR: direct immediate microscopic examination under an inverted microscope, immunocytochemistry of α-tubulin, and assay of the reduction of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

Tubulin was visualized in methanol-fixed cultures, essentially as previously described (24), after incubation with monoclonal anti-α-tubulin antibodies (1:1000 dilution), using an anti-murine IgG secondary antibody coupled to peroxidase (1:50 dilution), and diaminobenzidine-H_2O_2 for visualization. MTT reduction was determined by measuring spectrophotometrically the blue insoluble formazan product after one h incubation of the cultures with 150 μM MTT, as previously described for several types of cell cultures including neurons (25–27); formazan formation is an index of the functional state of the respiratory chain (28).

Statistical analyses were carried out using one-way ANOVA test.

RESULTS

Histology. Histological examination was carried out at 8, 16 and 24 h of incubation in the presence of 20, 50 and 100 μM RuR. The appearance of both neuronal and astroglial control cultures was similar to that previously described (22). As shown in Figs. 1 and 2, 4–6 DIV neuronal somata were well defined, possessed a clear halo, and formed large clusters joined by long, thin and even processes. On the other hand, 14–30 DIV as-
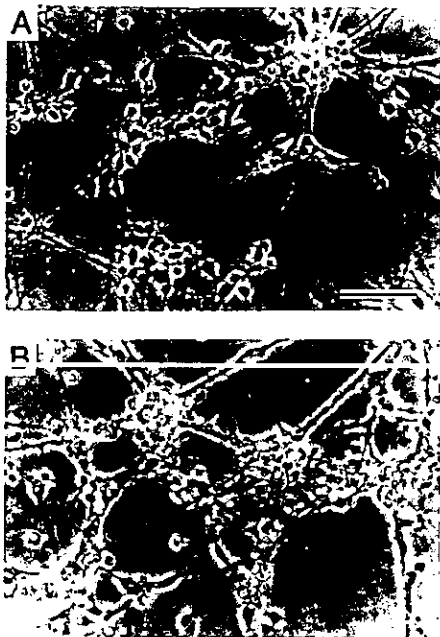
Fig. 1. Four-DIV cortical neurons incubated for 24 h in Locke medium in the absence (control, A) and in the presence of 100 μM RuR (B). Cultures treated with RuR showed a notable loss of apparently normal neurons and processes, as well as numerous varicosities in neurites. This damaging effect of RuR was observed in all the experiments used for determination of MTT reduction, in both cortical and cerebellar neurons (Fig. 4). Bar = 40 μm.
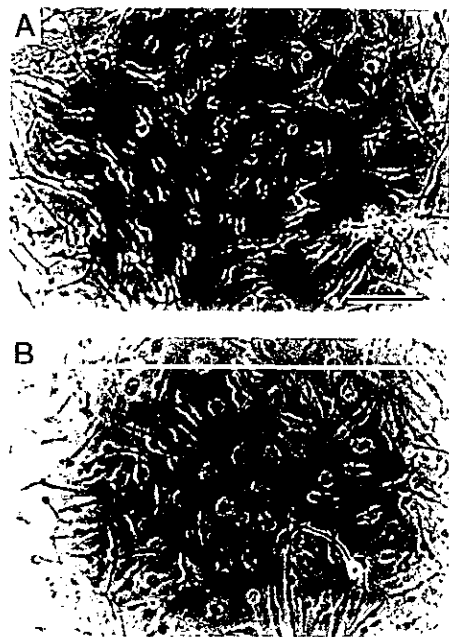
Fig. 2. Seventeen-DIV cerebellar astrocytes incubated for 24 h in Locke medium in the absence (control, A) and in the presence of 100 μM RuR (B). Only a few cells appear vacuolized in the RuR-treated culture. Bar = 80 μm.

trocytes showed few short processes and formed a monolayer.

In the neuronal cultures, the lowest concentration of RuR used (20 μM) did not produce any significant morphological alteration at any of the times studied, in spite of the fact that at 24 h RuR could be readily visualized inside some neuronal somata. With 50 μM and particularly with 100 μM RuR, a distinct staining of the somata occurred, which was most evident at 8 h of incubation and indicated the penetration of the dye. At 16 and 24 h some of the stain was lost, due to the destruction of the cells. Neuronal damage started to occur at about 16 h and it was obvious at 24 h, although some normally appearing cells containing no RuR were always observed. This damage was characterized by cell vacuolization and fragmentation and loss of neurites (Fig. 1).

In contrast to the above observations, with the exception of a few cells which looked vacuolized after 24

h with 100 μM RuR, no evident alteration in the morphology of cultured astroglia was observed at the RuR concentrations and incubation times tested (Fig. 2). Furthermore, in no instance was RuR observed inside the astrocytes.

Immunocytochemistry. Experiments on α-tubulin immunostaining were carried out in cortical neurons after 24 h of incubation with 100 μM RuR. Clear differences were observed between the cytoskeleton of control neurons and that of treated cultures. In the latter, neural processes appeared notably fragmented and detached from somata, suggesting disintegration of microtubules (Fig. 3).

Biochemistry. The MTT reduction assay allowed us to study more quantitatively the time and concentration dependency of RuR toxicity. As shown in Fig. 4, in 4–6 DIV cortical neurons treated with RuR the MTT reduction by living cells was curtailed as the concentration of RuR rose and as the exposure time was augmented. The cerebellar granule neurons showed a very similar behavior at the incubation times tested (8 and 24 hours, Fig. 4). On the other hand, cerebellar astrocytes were much
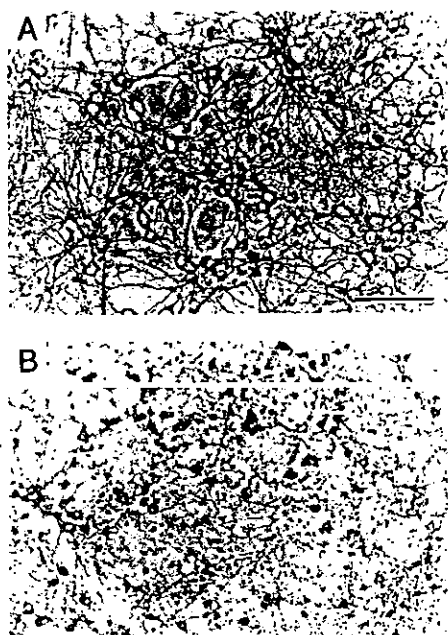
Fig. 3. Immunocytochemical staining of α-tubulin in 4-DIV cortical neurons incubated for 24 h in Locke medium in the absence (control, A) and in the presence of 100 μM RuR (B). Neurons form less clusters than those in Fig. 1 because they were seeded at about one-third cell density. RuR-treated neurons showed altered immunoreactive morphology of the somata, as well as detachment and notable fragmentation of neurites. Similar alterations were observed in 3 separate experiments. Bar = 40 μm.

Fig. 4. MTT reduction by 4-6-DIV cortical (A) and cerebellar (B) neurons after incubation with different RuR concentrations for 8, 16 or 24 h, as indicated. Results are expressed as percent of the control in the absence of RuR. Mean values of the number of experiments shown in parentheses ± SEM. *p < 0.05

more resistant to toxic effects of RuR than were the two neuronal types used. The only condition that resulted in effects statistically different from control was the incubation with 100 μM RuR during 24 h (Fig. 5).

Because in the above experiments cerebellar astrocytes were considerably older than neurons (14 vs 4–6 DIV), it was important to determine whether the neurotoxic effect of RuR was also present in older neurons. As can be seen in Fig. 6, the age of the neuronal cultures does not seem to influence significantly the toxic effect of RuR, since neuronal cells grown during 14 DIV were affected by the dye similarly to the younger neurons.

DISCUSSION

The present results demonstrate that in primary cell cultures RuR is able to penetrate neurons and produce cell damage, whereas glial cells are much more resistant.
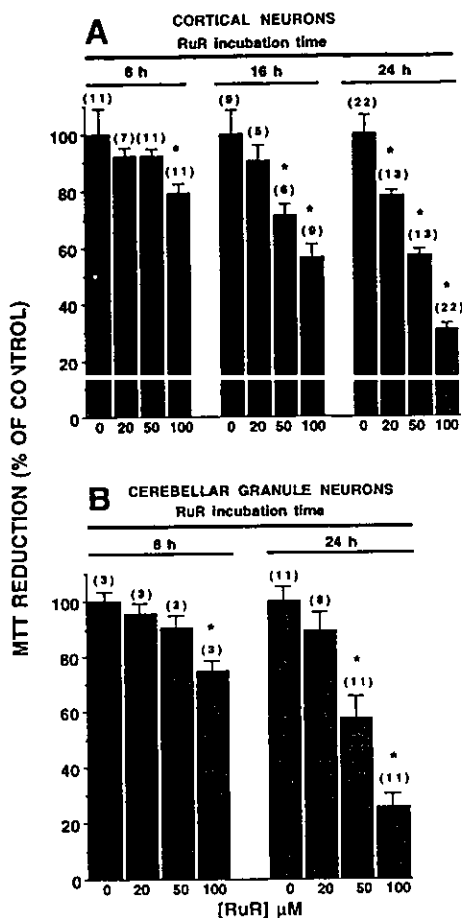
In fact, the dye was practically absent in cerebellar astrocytes, whereas it was readily observed inside neuronal somata. This difference between neurons and glia cannot be ascribed to the age of the cultures when RuR was added, since the same penetration and cell damage was observed in neurons cultured for 4–6 or for 14 DIV, which was the usual incubation period in the case of astrocytes. This selective localization was observed in both cortical and cerebellar granule neurons, suggesting that the capacity to incorporate RuR is absent in astro-
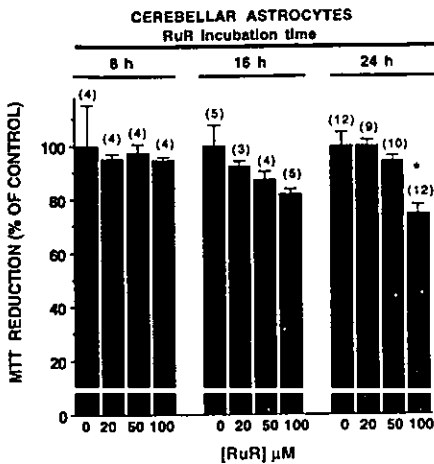
CEREBELLAR ASTROCYTES
RuR Incubation time

Fig. 5. MTT reduction by 14-30-DIV cerebellar astrocytes after incubation with RuR. Details as for Fig. 4.
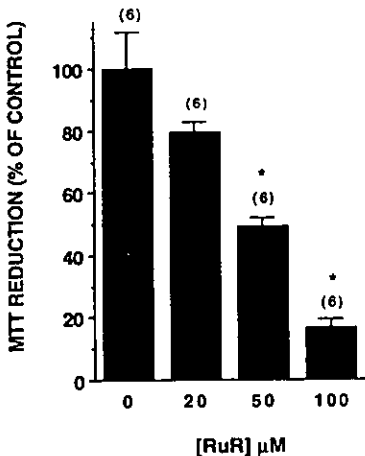

Fig. 6. MTT reduction by 14-DIV cortical neurons incubated with different concentrations of RuR for 24 h. Details as for Fig. 4.

cytes but it is shared by different types of neurons. Another interesting finding was that the RuR staining is restricted to neuronal somata, whereas neuronal processes are only slightly stained if at all. These characteristics of RuR incorporation into cells are surprisingly similar to the selective localization of the dye inside neurons after its microinjection into brain structures in vivo (16–18). Nevertheless, one important difference was that

whereas in vivo it is possible to see stained neurons in only a few minutes after the injection, in the primary cultures this required several hours. One possible explanation is that the cultures comprise immature neurons that do not necessarily reach the equivalent of adult age after a few DIV, at least in terms of RuR penetration.

Both the microscopic examination and the immunocytochemistry of α-tubulin showed that there was a progressive neuronal damage with time of exposure to RuR, as well as a dose-dependency of this toxic effect. It is interesting that not only the neuronal somata containing RuR were vacuolized, but also that neurites were fragmented and lost. In this respect, preliminary electron microscopic observations carried out in our laboratory confirmed the vacualization of those somata and revealed that it was restricted to the cells containing RuR, whereas some neurons of normal appearance were free of RuR.

A more precise quantitative comparison of the damage caused by RuR can be made with the MTT reduction assay, which is a valid indication of the number of living cells (25–28). The results of these experiments show that both cortical and cerebellar neurons are equally sensitive to the dye, because the dose-response curves are very similar, the rate of MTT reduction decreasing more than 70% after 24 h exposure to 100 μM RuR. The fact that practically no damage was observed at 8 h agrees with the morphological evaluation and suggests that the deleterious effect of RuR entails a slow process once the dye is inside the cells. This contrasts with the rapid effect of RuR when microinjected in the hippocampus in vivo, when clear damage was observed by electron microscopy in less than one h after the injection (18). This suggests either that the penetration of RuR is more rapid in vivo than in the cultures, or that in vivo the metabolic processes altered by RuR are more active and therefore the consequences of their derangement appear earlier.

It is interesting that in vitro RuR binds very quickly to synaptosomal membranes, and that there is no evidence, either in vitro or in vivo, that it penetrates or destroys the nerve endings (6,18). This strongly suggests that RuR toxicity depends on intracellular mechanisms occurring primarily in the somata. Therefore the most plausible explanation for the lack of damage of glial cells is the fact that RuR does not penetrate them, which suggests important functional differences between neuronal and glial membranes with respect to their permeability for RuR. At this time it is difficult to offer an explanation for the mechanism of RuR entrance into the somata. The dye is a polycationic compound of relatively high molecular weight (>500) and therefore the most probable way would be endocytosis. In living

amoeba RuR and its closely related compound, ruthenium violet, are very toxic and seem to bind irreversibly to the membrane and to be rapidly internalized by pinocytosis (29). This is now currently under investigation in our laboratory.

Several possibilities could account for the neurotoxicity of RuR. As mentioned in the Introduction, RuR might disrupt Ca^{2+} homeostasis and Ca^{2+}-related cell functions, due to the blocking of the binding of the cation to calmodulin or calsequestrin (12,13) and/or to the inhibition of its buffering by sarcoplasmic reticulum or mitochondria (1–4,14,15). This would result in an increased cytoplasmic Ca^{2+} concentration, which is a well established causative factor of neuronal death (30). The primary event in RuR toxicity could also be an alteration of mitochondrial oxidative function (2), which would cause derangement of energy metabolism. Finally, the disruption of the cytoskeletal structure and axoplasmic transport might also be involved in RuR neurotoxicity, as indicated by the loss of α-tubulin immunoreactivity observed in the present experiments. However, it is difficult to know whether this is a primary event or occurred as the consequence of alterations of Ca^{2+} homeostasis or energy metabolism. In any case, RuR seems to be a relevant tool for studying the intracellular mechanisms of neuronal damage, and neuronal culture offers a good experimental model for this purpose.

ACKNOWLEDGMENTS

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Ruthenium Red Neurotoxicity and Interaction With Gangliosides in Primary Cortical Cultures

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Ruthenium red (RR) is an inorganic polycationic dye able to exert several effects on the nervous system, including neurodegeneration, both in vivo and in cell cultures. Gangliosides have been shown to protect cultured neurons against several damaging conditions, and it has been postulated that RR can interact with the negative charges of the sialic acid residues of these molecules. In the present work we have tested the effect of the trisialoganglioside GT1b and the monosialoganglioside GM1 on the RR-induced neuronal damage in primary cortical cultures, as well as on the binding of RR to synaptosomes. GT1b at 100–200 µM concentrations partially protected against RR-induced neurodegeneration, as judged by light microscopy and by measurement of the reduction of a tetrazolium salt, while GM1 was ineffective. GT1b, but not GM1, also partly blocked both RR binding and its diminution in the culture medium occurring during incubation. These results suggest that the three negative charges of GT1b enable it to interact with RR and as a consequence the entrance of the dye into the cells is blocked and neurotoxicity is diminished, although other mechanisms of protection cannot be excluded. Endogenous polysialic acid–containing molecules do not seem to be involved in RR effects, since the removal of sialic acid residues by treatment with neuraminidase did not prevent the cell damage. J. Neurosci. Res. 49:72–79, 1997. © 1997 Wiley-Liss, Inc.

Key words: ruthenium red; neurodegeneration; neuronal cultures; neuraminidase

INTRODUCTION

The polycationic inorganic dye ruthenium red (RR) exerts several effects on neuronal function, such as the blockade of voltage-sensitive calcium channels, inhibition of neurotransmitter release, hyperexcitation, and neurodegeneration. RR binds to Ca^{2+} sites on the synaptosomal membrane (Massieu and Tapia, 1988), blocks Ca^{2+} transport in mitochondria (Moore, 1971; Broekemeier et al., 1994), and synaptosomes (Tapia et al., 1985b; Arias and Tapia, 1986; Taipale et al., 1989; Hamilton and Lundy, 1995), and inhibits the Ca^{2+}-dependent release of neurotransmitters (Tapia and Meza-Ruiz, 1977; Tapia et al., 1985a,b; Hamilton and Lundy, 1995).

Besides the above effects in vitro, when RR is microinjected in the rat or cat cerebral ventricles or parenchyma it induces intense behavioral and electrophysiological hyperexcitation, as well as notable neuronal damage of the injected region (Tapia et al., 1976; Tapia and Flores-Hernández, 1990; García-Ugalde and Tapia, 1991; Belmar et al., 1995). These studies in vivo showed that RR is able to penetrate into neuronal somata, where it produces vacuolization and cell destruction, without any apparent neuropil or glial cell damage. This selectivity for neurons was demonstrated to occur also in primary cortical and cerebellar primary cultures. In such cultures, incubation with 20–100 µM RR for 8–24 hr resulted in the entry of the dye into neurons and subsequent neuronal damage, while astrocyte cultures were not affected (Velasco et al., 1995).

Gangliosides are sialic acid-containing glycolipids abundant in neuronal plasma membrane. Among the most prominent gangliosides are the monosialoganglioside GM1 and the trisialoganglioside GT1b (Yu and Saito, 1989). These compounds possess several interesting properties, including a protective action in cultured neurons against a variety of damaging conditions, such as trophic factor deficiency (Ferrari et al., 1993), hypoglycemia (Facci et al., 1990), excitotoxicity due to glutamate receptor activation (Favaron et al., 1988; Manev et al., 1990; Kikuchi and Kim, 1993), and massive calcium influx (Nakamura et al., 1992). With regard to RR, it has been postulated that the dye interacts directly with sialic acid residues of gangliosides (Luft, 1971), and it has been shown that removal of sialic acid by treatment with neuraminidase (NAase) results in a diminished inhibitory effect of RR on neurotransmitter release (Baux et al., 1979; Wieraszko, 1986).

The above data led us to postulate that gangliosides might play a role in the entrance of RR into neurons and therefore in its neurotoxicity, and the aim of the present work was to test such hypothesis. For this purpose, we have studied whether exogenous gangliosides can protect cultured neurons from RR-induced degeneration and whether this can be related to a RR–ganglioside interaction. In addition, we treated the cultures with NAase in order to test if the sialic acid residues of endogenous gangliosides or sialoproteins were necessary for the RR-induced neurotoxicity.

MATERIALS AND METHODS

Cell Culture

Cortical neuronal cultures were prepared as previously described (Velasco et al., 1995). Briefly, the cortices of 17-day-old Wistar rat fetuses were dissociated by trypsinization and the cells (1.25–1.5×10^6/ml or, in some experiments, 0.6–0.7×10^6/ml) were seeded in plastic dishes (Costar, Cambridge, MA) previously coated with poly-L-lysine, in basal medium Eagle (Sigma Chemical Co., St. Louis, MO) supplemented with 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 10% fetal bovine serum (Gibco, Gaithersburg, MD). The cultures were maintained in a 5% CO_2-95% air atmosphere at 37°C. Non-neuronal proliferation was curtailed by 10 µM cytosine arabinoside added at 20 hr after seeding.

Exposure to RR and Gangliosides

Cultures were maintained for 4–6 days in vitro before they were exposed to RR (Sigma) in Locke's medium (0.5 ml), as previously described (Velasco et al., 1995). RR was present in the cultures for 16 or 24 hr, at the concentrations indicated in Results. All experiments were made in duplicate and included control dishes with RR-free Locke's medium. Two gangliosides, GM1 and GT1b (from bovine brain, Sigma), were tested for neuroprotection at the concentrations indicated in Results. Gangliosides were coincubated with RR during 16 or 24 hr or, in the case of GT1b, preincubated with the cultures. In the latter experiments, GT1b was present either for 2 hr in Locke's medium before the addition of 100 µM RR, or for 24 hr in culture medium, prior to 24-hr incubation in Locke's medium which contained RR with or without the ganglioside. In order to avoid oxidation of the gangliosides, they were dissolved in a 2:1 chloroform-methanol mixture, the solvents were subsequently evaporated with nitrogen, and final solutions were prepared in water thoroughly bubbled with nitrogen (Toffano et al., 1980). To test whether the effects of GT1b were due solely to its negative charges, the polyanionic molecule pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt; Molecular Probes, Eugene, OR) was also comparatively studied.

Evaluation of Cell Damage

The neurotoxic effect of RR under the different experimental conditions tested was evaluated by phase-contrast light microscopy and by spectrophotometric assay of the mitochondrial reduction of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma), as previously described (Velasco et al., 1995). This reaction assesses the functional state of the respiratory chain (Mossman, 1983).

Neuraminidase Treatment

Cultures were incubated with 0.2 U/ml of NAase (EC 3.2.1.18; from V. cholerae, Sigma) in basal medium Eagle for 3 hr, as described by Wu and Ledeen (1991). Higher concentrations of NAase could not be used because they produced cell damage, as assessed by light microscopy examination and MTT reduction. At the end of the incubation period the cultures were washed once with Locke's medium and exposed to RR in fresh medium, as described above. In order to ascertain the activity of NAase under the experimental conditions described, in some experiments the concentration of sialic acid in the culture medium was measured after enzyme treatment by the spectrophotometric thiobarbiturate procedure described by Warren (1959). Sialic acid standard was from Sigma.

RR–Ganglioside Interaction

The possible direct interaction of gangliosides with RR in the extracellular medium was studied by measuring the effect of GM1 and GT1b on the binding of RR to synaptosomes. Rat forebrain synaptosomes were prepared by the method of Löscher et al. (1985), and RR binding was determined, after incubation for 15 min at room temperature with different concentrations of RR, by the procedure previously described (Tapia et al., 1985a). Control and experimental tubes containing gangliosides or pyranine, at the concentrations indicated in Results, were handled in parallel. At the end of the incubation period, synaptosomes were centrifuged in a microfuge, the RR concentration in the supernatant was quantified spectrophotometrically at 534 nm, and the fraction bound to the synaptosomes was calculated by difference from a control value without synaptosomes (Tapia et al., 1985a). The presence of GM1, GT1b, or pyranine did not interfere with the absorbance of RR at 534 nm. Synaptosomal protein was determined by the method of Bradford (1976), with bovine serum albumin as a standard.

To evaluate the extent of penetration of RR into neurons in culture, the spectrophotometric method described above was used to measure the concentration of the dye in the culture medium after 24 hr incubation. The effect of gangliosides on this parameter was also assessed, by measuring RR concentration after coincubation with GM1 and GT1b.

Statistical analyses were carried out using analysis of variance (ANOVA) test.

RESULTS

Protection by Exogenous Gangliosides

At both high and low seeding cell density, cultured cortical neurons growed in clusters and developed a network of thick processes. As previously reported (Velasco et al., 1995; Tapia and Velasco, 1997), the cultures showed signs of degeneration when exposed to 20–100 µM RR for 24 hr. The most prominent alterations observed under the microscope were the appearance of multiple varicosities in the neurites, as well as their fragmentation and loss (Fig. 1B). Biochemically, a notable decline in the capability for reducing MTT was observed (Fig. 2). Coincubation with GT1b for 24 hr resulted in partial protection against this RR-induced damage, as assessed by both light microscopy (Fig. 1C) and MTT reduction. At the three concentrations of GT1b tested (100, 150, and 200 µM), the extent of recovery of MTT reduction, in absolute values, was similar (about 30%), independently of the RR concentration. However, the percent recovery relative to the damage (i.e., when the percent decrease of MTT reduction with RR alone = 100%) was inversely proportional to the RR concentration (74%, 62%, and 36% average recovery with 20, 50, and 100 µM, respectively, Fig. 2). A similar protection by GT1b was also found when the incubation period with RR was 16 hr (not shown). In some experiments higher GT1b concentrations (500 µM and 1 mM) were tested, but it was observed that in control cultures without RR the cells detached themselves from the dish bottom and looked damaged under the microscope.

Preincubation with 100 µM GT1b in Locke medium for 2 hr did not improve the protection against 100 µM RR (24% recovery, n = 4). When 200 µM GT1b was preincubated for 24 hr in culture medium and it was also present in the RR-containing Locke medium, again no additional protection was observed, as compared to the above-described coincubation experiments (65%, 30%, and 29% recovery with 20, 50, and 100 µM RR, respectively, n = 2). Finally, no protection at all was observed when GT1b was preincubated for 24 hr and then removed from the RR-containing Locke medium (n = 2), suggesting that free ganglioside in the medium must interact with RR in order to prevent its toxic effect.

In contrast to GT1b, GM1 did not affect the RR-induced neurodegeneration, even when present at a tenfold higher concentration than RR (Table I). Similarly, the trivalent anion pyranine, at 50 or 100 µM concentration, failed to protect against RR-induced neurotoxicity (Table I).

Effects of NAase

As shown in Figure 3A, treatment of the cultures with NAase did not prevent the damage produced by 20–100 µM RR. This lack of effect was also apparent by microscopic examination of the cultures (not shown). That the enzyme was active under the experimental conditions used is shown by the remarkable increase in the concentration of free sialic acid in the medium as compared to the negligible control levels (Fig. 3B).

Ganglioside–RR Interaction

As shown in Figure 4, 50 µM GT1b inhibited 30% the binding of 50 µM RR to synaptosomes, and this inhibition increased to 60% when RR concentration was fivefold lower. In contrast, neither GM1 nor pyranine affected RR binding (Fig. 4).

The results of RR measurement in the culture medium are shown in Figure 5. In the absence of gangliosides, at the three concentrations of the dye tested about 50% of the initial RR remains in the medium after 24 hr incubation. Similarly to the inhibition of synaptosomal binding, coincubation with GT1b significantly increased the concentration of RR in the medium. The increase was similar with 100, 150, and 200 µM of the ganglioside (differences were not statistically significant), but it was inversely proportional to the initial concentration of RR. Increments were 93–119%, 53–75%, and 23–29% with 20, 50, and 100 µM RR, respectively (Fig. 5). In contrast to GT1b, GM1 did not significantly affect the concentration of RR in the culture medium (Table II).

DISCUSSION

The present results show that GT1b, but not GM1, partially prevented the RR-induced neuronal damage in cortical cultures. With 100–200 µM GT1b the protection was nearly complete at the lowest RR concentration used and decreased at higher dye levels. Concentrations of GT1b higher than 200 µM, which might be more effective, could not be used because they caused disruption of the cultures. Three negative charges seem to be required for this protection, since the single sialic acid–containing ganglioside GM1 was ineffective. However, the lack of effect of pyranine, a nonganglioside molecule containing also three negative charges, suggests that the three-
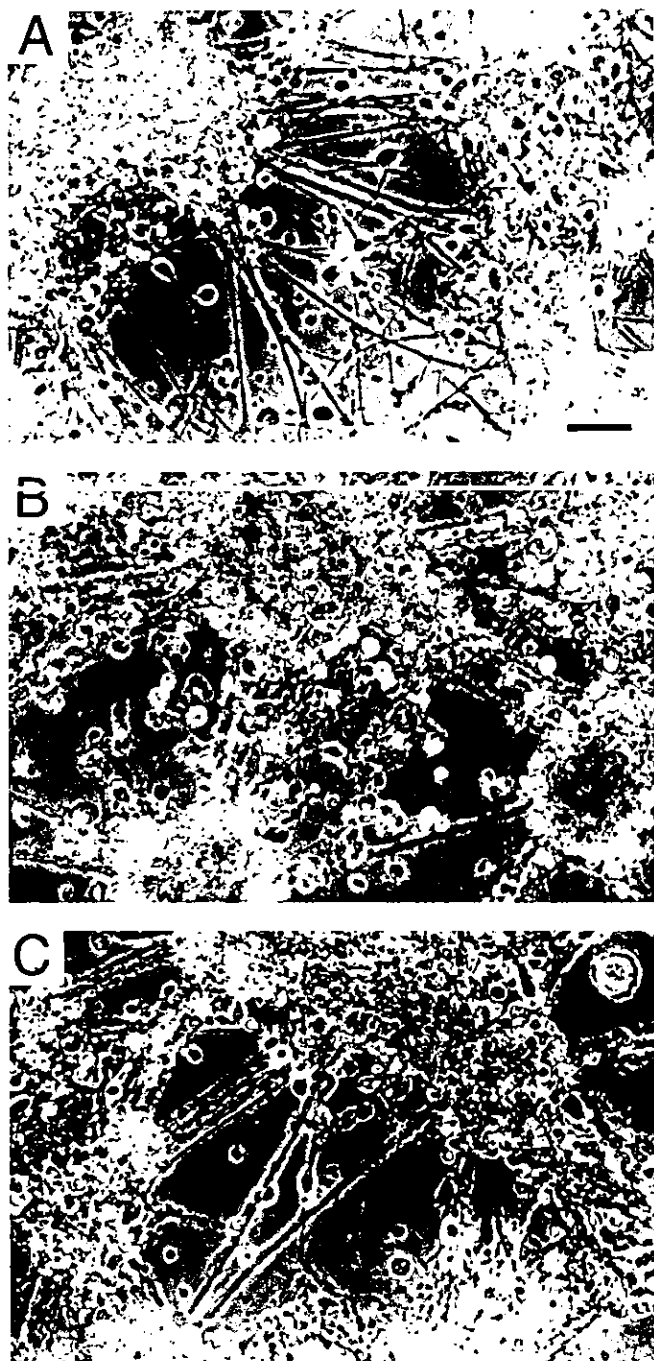
Fig. 1. Phase-contrast micrographs of control and RR-treated neuronal cultures. A: Control, showing cell clusters connected by numerous uninterrupted neural processes, as well as scattered dark neuronal somata surrounded by a bright halo. B: Cultures treated with 100 μM RR for 24 hr; few normal-looking neuronal somata can be seen, and neurite loss is apparent; the remaining neurites look thinner, fragmented, and with varicosities. C: Cultures treated with 100 μM RR and 100 μM GTlb; protection is evident by the presence of dark neuronal somata and by the preservation of numerous neural processes. Bar = 35 μm.
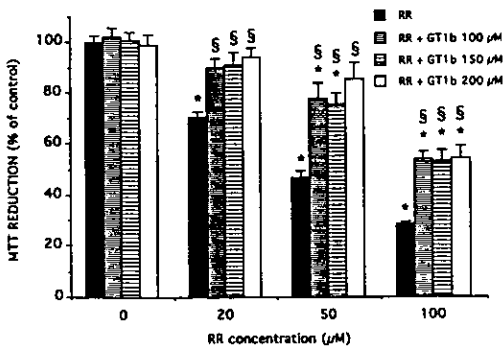
Fig. 2. Effect of GT1b on neuronal damage induced by 24-hr incubation with different RR concentrations, as assessed by MTT reduction. *, $P < .05$ relative to control without RR; §, $P < .05$ relative to RR alone; n = 6–10.

TABLE I. Lack of Protective Effect of GM1 and Pyranine Against RR-Induced Neurodegeneration, as Assessed by MTT Reduction After 24-hr Incubation With the Dye at the Concentrations Indicated[a]

RR concentration (μM)	0	20	50	100
No addition	100.0 ± 7.0	70.6 ± 2.1	46.5 ± 2.8	28.6 ± 0.9
GM1 100 μM	97.9 ± 5.7	70.7 ± 10.4	42.9 ± 2.8	29.9 ± 4.1
GM1 150 μM	101.9 ± 4.2	67.7 ± 9.2	36.4 ± 2.1	27.9 ± 0.7
GM1 200 μM	103.2 ± 4.2	78.5 ± 7.3	40.5 ± 0.3	30.1 ± 2.2
Pyranine 50 μM	98.2 ± 5.7	—	—	29.1 ± 3.1
Pyranine 100 μM	94.0 ± 0.6	—	—	21.8 ± 2.7

[a]Figures are percent of MTT reduction control values (cultures without RR). Means ±SEM for four independent experiments. All values with RR are significantly different from the controls without RR ($P < .05$).
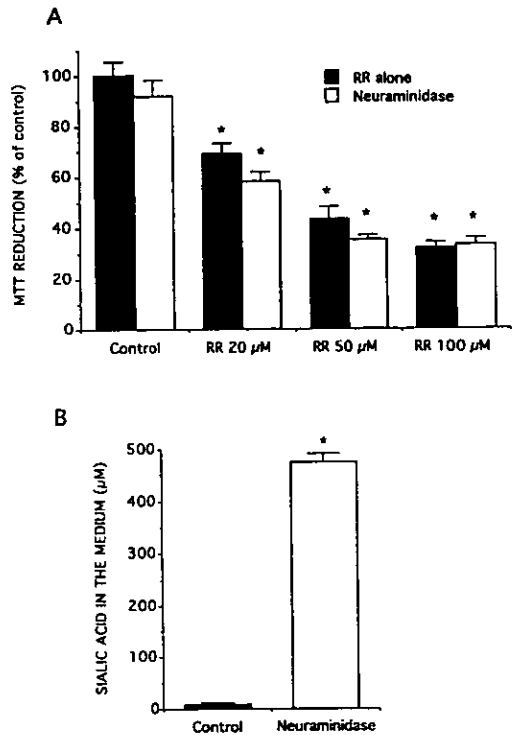

Fig. 3. Action of NAase on neuronal cultures. A: Lack of effect of the enzyme on neuronal damage induced by 24-hr incubation with different RR concentrations, as assessed by MTT reduction. B: NAase activity shown by the release of sialic acid to the medium. *, $P < .05$ relative to control; n = 4.

dimensional arrangement of the charges present in GT1b is important for the protection.

A good correlation was established between the protective action of GT1b and its interaction with RR: This ganglioside clearly inhibited the binding of RR to the synaptosomal membrane, whereas neither GM1 nor pyranine had any effect. Furthermore, in the cultures exposed to RR in the presence of GT1b, those cells that looked undamaged were not stained by the dye. Therefore, the most probable explanation for the protection by the polysialic ganglioside is that it combines with RR and as a consequence reduces the amount of free dye available to penetrate into the cells. In accord with this interpretation, when GT1b was preincubated for 24 hr to allow its incorporation into cell membranes (Saqr et al., 1993) and was then removed from the medium, no protection was observed, which suggests that the ganglioside needs to be present in the RR-containing medium in order to protect the cells. The results of RR measurement

in the culture medium support this conclusion, as will be discussed next.

Since RR is not metabolized, the difference between the concentration of RR added to the cultures and that present at the end of the incubation period may be used to estimate indirectly the amount of the dye taken up by the neurons. As shown in Figure 6, when this calculation is carried out at the different RR concentrations used and the resulting values are plotted against the cell damage produced by the drug, a nearly linear correlation between the two parameters is found (control curve with RR alone). This is consistent with the above conclusion regarding the relationship between intracellular RR and neuronal damage. Furthermore, similarly to its inhibitory effect on RR binding to synaptosomes, incubation with GT1b resulted in an increase in the concentration of RR in the medium, and therefore the plot cell damage-intracellular RR was shifted to the left in the
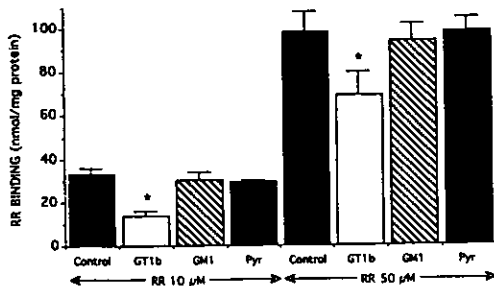
Fig. 4. Effect of 50 μM gangliosides and pyranine on the binding of 10 and 50 μM RR to synaptosomes. *, $P < .05$ relative to control; n = 3–12.
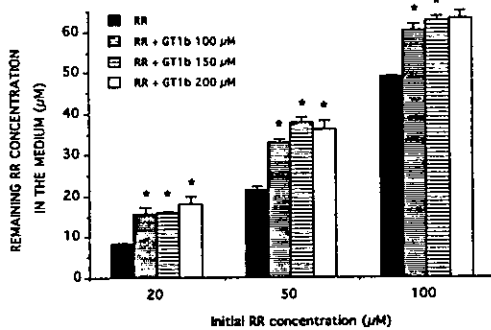

Fig. 5. Concentration of RR in the culture medium after 24-hr incubation, and effect of GT1b. *, $P < .05$; n = 3.

TABLE II. Lack of Effect of GM1 on RR Levels in the Culture Medium After 24 hr Incubation With the Dye at the Concentrations Indicated*

RR initial concentration (μM)	20	50	100
No addition	8.2 ± 0.32	21.6 ± 0.78	49.0 ± 0.27
GM1 100 μM	9.7 ± 0.53	27.5 ± 4.3	52.5 ± 6.7
GM1 150 μM	10.1 ± 1.3	25.5 ± 4.0	51.2 ± 1.3
GM1 200 μM	10.9 ± 0.72	23.5 ± 3.8	54.0 ± 5.0

*Figures are μM concentrations of RR at the end of the incubation. Means ±SEM for three independent experiments. None of the values was significantly different from the controls without GM1.
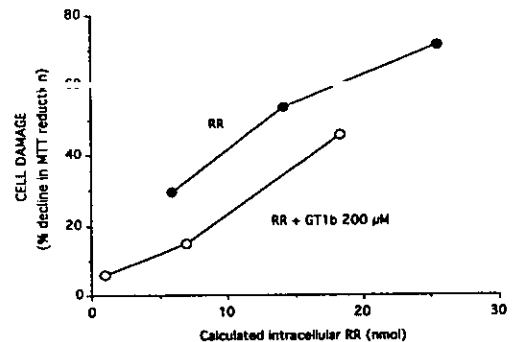

Fig. 6. Neuronal damage as a function of intracellular RR levels, in the absence and in the presence of GT1b. The values of the abscissa were calculated from the data of Figure 5 at the three RR concentrations tested, as described in the text, and those of the ordinate from the data of Figure 2. In each curve the first, second, and third points correspond to the incubation with 20, 50, and 100 μM RR, respectively.

presence of the ganglioside (Fig. 6). It should be noted, nevertheless, that in the presence of GT1b a similar or even higher calculated intracellular RR produced less damage than in the absence of the ganglioside, which suggests the involvement of other mechanisms of protection besides the trapping of RR.

One of these possible additional mechanisms is an inhibition of protein kinase C (PKC), since an activation of this enzyme has been related to neurotoxicity. In fact, it is known that gangliosides, particularly GT1b, inhibit PKC, both in cell-free systems (Kreutter et al., 1987; Katoh, 1995) and when activated by glutamate receptor agonists (Vaccarino et al., 1987; Manev et al., 1990; Costa et al., 1994). Other possible mechanisms of protection by gangliosides are the inhibition of Ca^{2+}-activated NO synthase (Dawson et al., 1995) or a facilitation of the homeostatic mechanisms of intracellular Ca^{2+} concentration, as it has been shown after ionophore- or glutamate-induced increases of cytoplasmic Ca^{2+} (de Erausquin et

al., 1990; Nakamura et al., 1992; Wu and Ledeen, 1994). Consistent with the latter possibility is the observation that intracellular RR interferes with intraneuronal Ca^{2+} homeostasis, delaying the return to normal cytoplasmic levels after elevations induced by caffeine or electrically induced depolarization (Thayer and Miller, 1990; Marrion and Adams, 1992).

That RR must penetrate the cells to induce neurodegeneration has been previously shown, both in vivo and in neuronal cultures (García-Ugalde and Tapia, 1991; Belmar et al., 1995; Velasco et al., 1995; Tapia and Velasco, 1997). Since RR is a relatively large molecule (molecular weight ~550) and is highly charged, the mechanism of this penetration is difficult to understand. RR has been observed inside live amoeba, and it has been suggested that it entered the cells by endocytosis (Szubinska and Luft, 1971). However, we have been unable to find endocytotic images in electron microscope examina-

tion of our RR-treated cultures (Velasco and Tapia, unpublished).

As discussed above, the presence of polysialic residues in GTlb seems to be necessary to bind RR and to protect against its toxicity. NAase cleaves sialic acid residues of sialoproteins and di- and trisialogangliosides, but steric hindrance prevents the hydrolysis of monosialogangliosides, which therefore remain as the final product (Schauer et al., 1980; Wu and Ledeen, 1991). Therefore, the lack of protective action of NAase treatment against RR-induced neurodegeneration suggests that either an interaction of RR with plasma membrane sialic acid residues is not a previous step for its internalization, or that, differently from its behavior in solution, the dye interacts with endogenous monogangliosides and in this way it enters the cell and induces damage.

In conclusion, the present study confirms that internalization of RR into neurons is necessary for the induction of neurodegeneration and shows that the damage can be partially blocked by GTlb but not by other polyanions or by monosialogangliosides. The protection by the polysialoganglioside seems to involve both the trapping of RR and additional intracellular mechanisms, such as PKC inhibition or correction of Ca^{2+} dyshomeostasis.

ACKNOWLEDGMENTS

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MECHANISMS OF RUTHENIUM RED NEUROTOXICITY IN PRIMARY CORTICAL CULTURES

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Abbreviations used: RR, ruthenium red; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DMSO, dimethyl sulfoxide; BAPTA, *bis*-(o-aminophenoxy)-ethane-N,N,N',N'-tetracetic acid; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo-(a,d)cyclohepten-5,10-imine maleate; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline.

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ABSTRACT

Ruthenium red (RR) is a polycationic dye able to induce neuronal death *in vivo* and in primary cultures. In this work, we have analyzed the ultrastructural alterations induced by RR in rat cortical neuronal cultures using both scanning and transmission electron microscopy. We also examined the involvement of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$), and of the membrane Ca^{2+}-binding sites, in the neurotoxicity of the dye. Since internalization of RR into cultured neuronal somata seems to be a requisite to induce neurodegeneration, the possibility that RR interfere with the normal mitochondrial function, in isolated brain mitochondria was also tested. Whether RR can induce apoptosis, and the role of ionotropic glutamate receptors in the RR-induced death were investigated. The results indicate that RR produced a progressive damage of neurites and plasma membrane after 8-24 h, and remained associated with the nucleus of affected neurons. No damage or introduction of RR was evident in neurons with intact plasma membrane. RR caused higher resting $[Ca^{2+}]_i$ and an exacerbated cytoplasmic Ca^{2+} elevation after depolarization. However, compounds capable to prevent $[Ca^{2+}]_i$ increases had no effect on RR toxicity. La^{3+}, an ion that displace RR bound to synaptosomes, did not prevent but potentiated RR-induced death, possibly because of La^{3+} alone caused neurodegeneration. Mitochondrial function was significantly diminished after treatment of isolated brain mitochondria with RR. We did not observe modification of RR toxicity with glutamate receptor antagonists. Apoptotic images were not observed, and protein synthesis inhibitors could not reduce the neuronal death

promoted by RR, suggesting that the dye induces necrosis. The results of this works support the hypothesis that RR is exerting its damaging action from inside the cells, probably by interfering with mitochondrial function.

Running title: Mechanisms of ruthenium red toxicity.

The inorganic polycationic dye ruthenium red (RR) is frequently used to study the role of Ca^{2+} in cell function. Besides its well known Ca^{2+} blocking properties in mitochondria (Vasington et al., 1972; Reed and Bygrave, 1974; Sparagna et al., 1995) and ryanodine receptors (Mézsaros and Volpe, 1991; Ma, 1993), RR inhibits the Ca^{2+}-dependent neurotransmitter release in synaptosomes (Tapia and Meza-Ruiz, 1977; Tapia et al., 1985; Hamilton and Lundy, 1995) and neuromuscular junctions (Person and Kuhn, 1979; Hamilton and Lundy, 1995). In these preparations, RR seems to prevent Ca^{2+} flux mainly through N and P type channels (Massieu and Tapia, 1988; Hamilton and Lundy, 1995).

When RR is injected intracerebrally, it induces intense electrical and behavioral signs of hyperexcitation, as well as neurodegeneration (Tapia et al., 1976; Tapia and Flores-Hernández, 1990; García-Ugalde and Tapia, 1991; Belmar et al., 1995). The RR-induced neuronal death has also been observed in primary cultures (Velasco et al., 1995; Velasco and Tapia, 1997; for a review of the actions of RR in the nervous system, see Tapia and Velasco, 1997). We have suggested that both in vivo and in cultures, the penetration of RR into neurons is a necessary step prior to induce degeneration, based on the following observations: RR can be seen inside the neuronal somata before there is any obvious alteration of cell morphology (Belmar et al., 1995; Velasco et al., 1995); in astroglial cultures, RR did not penetrate cells neither produce significant cell death (Velasco et al., 1995); when RR uptake into neurons was reduced, its neurotoxicity was also diminished (Velasco and Tapia, 1997).

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The intracellular actions of RR have been studied by some groups after introducing the dye to neurons, mainly to test whether cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) is modified. Thus, it has been shown that in isolated bull-frog sympathetic neurons RR potentiated and prolonged the response of the cells to caffeine (a promoter of Ca^{2+} release from endoplasmic reticulum), without affecting basal Ca^{2+} levels (Marrion and Adams, 1992). A similar inhibition of Ca^{2+} buffering by RR was observed in isolated rat dorsal root ganglion neurons, after depolarization-induced $[Ca^{2+}]_i$ elevations (Thayer and Miller, 1990). In contrast, in cerebellar slices, RR completely abolished the $[Ca^{2+}]_i$ response to caffeine in Purkinje neurons (Kano et al., 1995).

The aim of the present study was to know in more detail the alterations caused by RR in neurons, and how they relate to degeneration in primary cortical cultures. We used morphological and biochemical approaches, such as electron microscopy, $[Ca^{2+}]_i$ measurements, and evaluation of mitochondrial function in cells and in isolated mitochondria, after RR treatment. All these parameters have been related to neurodegeneration and our present results show that RR is capable of altering them.

EXPERIMENTAL PROCEDURES

Neuronal cultures

Rat cortical cultures were prepared as described (Velasco et al., 1995; Velasco and Tapia, 1997). Briefly, the cerebral cortex of 17 days fetuses were trypsinized and plated (260,000-310,000 cells/cm^2) in plastic dishes (Costar, Cambridge, MA) or glass

coverslips (Fisher, Pittsburgh, PA) coated with poly-L-lysine, with basal medium Eagle's (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, MD), 2 mM glutamine and penicillin (50 U/mL)/streptomycin (50 µg/mL). 10 µM cytosine arabinoside was added 24 h after seeding to curtail astrocytic proliferation. The cells were maintained in a humidified incubator with 5% CO_2-95% air at 37°C during 4-6 days before use.

Treatment with RR and evaluation of mitochondrial function

Neuronal cultures were exposed to 0, 20, 50 and 100 µM RR as described (Velasco et al., 1995; Velasco and Tapia, 1997). Briefly, the Eagle's medium was substituted by Locke's medium (composition: NaCl 154 mM, KCl 5.6 mM, $CaCl_2$ 2.3 mM, $MgCl_2$ 1.2 mM, $NaHCO_3$ 3.6 mM, glucose 5.6 mM, HEPES 5 mM, pH 7.4) . The RR-treated cells were returned to the incubator for the period of time indicated under Results. In some experiments, the mitochondrial function of neurons was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Velasco et al., 1995; Velasco et al., 1996; Velasco and Tapia, 1997). Briefly, MTT (150 µM) was added to the cultures and incubated at 37°C for 1 h. The formazan product was solubilized with isopropanol and quantified spectrophotometrically at 570 nm.

We also evaluated the functional state of isolated brain mitochondria (see below) in the absence and presence of 100 µM RR. 5 mM malate, 5 mM glutamate, 0.25 mM ADP and 150 µM MTT were added to the mitochondrial suspension for 15 min at 37°C, and the formazan production was quantified at 570 nm after

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centrifugation (10 000 g during 3 min at room temperature) and solubilization (with isopropanol) of the mitochondrial pellet. Results are expressed, like in the case of cell survival evaluation, as percent of control (RR-free condition).

When the effect of Ca^{2+} chelators and of other drugs affecting Ca^{2+} was tested, the compounds were added to the cultures at the concentrations indicated in Results, immediately prior to RR or, in some cases, 2 h before RR.

Electron microscopy

After treatment with RR, cultures were washed and fixed with 3% glutaraldehyde in cold phosphate buffered saline (pH 7.4) and processed for either scanning or transmission electron microscopy. For scanning microscopy, coverslips were postfixed in OsO_4, dehydrated with ethanol (30 to 100%), dried to critical point with liquid CO_2 and covered with gold and observed in a Jeol JSM 5410LV microscope. For transmission microscopy, cells were postfixed with 2% OsO_4, dehydrated, included in propyl oxide, and ultrathin sections were stained with lead citrate and uranyl acetate and observed in a Jeol 1200 EXII microscope.

Measurement of intracellular Ca^{2+}

The $[Ca^{2+}]_i$ was determined using Fura 2 (Grynkiewicz et al., 1985). Neurons in culture were loaded in Eagle's medium with 10 μM Fura 2-AM, dissolved from a 1 mM stock in dimethyl sulfoxide (DMSO), for one h at 37°C, and then treated/untreated with 100 μM RR in Locke's solution. After the times indicated in Results, cells were transferred in Locke's medium to a 500 μL perfusion chamber and $[Ca^{2+}]_i$ was determined at room temperature, using an image system composed

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of a Diaphot inverted microscope (Nikon, Tokyo, Japan), a spectrofluorometer as the 340/380 nm excitation source (SLM Aminco, Rochester, NY), a 510 nm emission filter, an intensifying camera (Hamamatsu, Shizuoka, Japan), and an Image 1/FL software (Universal imaging, West Chester, PA). We estimated the basal $[Ca^{2+}]_i$ and the change produced by depolarization with high K^+ concentration, in the absence and in the presence of 100 µM RR. To study the effect of K^+ depolarization, Locke's medium was rapidly substituted by an isosmotic medium containing 100 mM KCl. In the coverslips treated with RR, we observed some cells that looked red in light microscopy. These neurons had high $[Ca^{2+}]_i$ and after some time they lost their fluorescence, presumably because of death. We selected 6-14 cells for simultaneous measurement in a 100X field, including when possible those with RR inside, and averaged the $[Ca^{2+}]_i$ of all neurons. Calibration was made by adding the Ca^{2+} ionophore ionomycin (5 µM) to increase the $[Ca^{2+}]_i$ to a maximum value, and 20 mM EGTA to reach the minimum Ca^{2+} level. $[Ca^{2+}]_i$ was obtained by using the following formula: $[Ca^{2+}]_i = K_D B (R-R_{min})/(R_{max}-R)$, where K_D was 225 nM, B the 380 nm intensity ratio of zero Ca^{2+}/saturating Ca^{2+} concentration, R is the measured 340 nm/380 nm ratio and R_{min} and R_{max} are the 340/380 ratios with minimum and maximum Ca^{2+} concentrations, respectively (Grynkiewicz et al., 1985). In order to assess the possible interference of RR with the Fura 2 method, we measured the 340/380 nm ratio in solutions containing Fura 2 and Ca^{2+} concentrations enough to give R_{min} and R_{max}, in the absence and presence of 100 µM RR. We did not observe any modification in the fluorescence ratios in the presence of the dye. Since it has

been reported that Fura 2 is not an accurate probe to measure $[Ca^{2+}]_i > 1 \mu M$ (Hyrc et al., 1997), we discarded the cells reaching this concentration.

Mitochondrial isolation

A crude mitochondrial fraction was obtained as described by Löscher et al. (1985) from adult male Wistar rats. Briefly, whole forebrain was homogenized in 0.32 M sucrose and centrifuged at 1 000 g for 10 min at 4°C. The supernatant was layered over 1.2 M sucrose and centrifuged at 220,000 g during 15 min at 4°C. The mitochondrial pellet was resuspended in a medium containing 180 mM KCl, 5 mM Tris, 1 mM $MgCl_2$, 0.5% bovine serum albumin, pH 7.2 and mitochondrial function was determined as described above.

Statistics

All experiments were carried out at least 3 times. Experiments were considered independent when we used different cultures or, for isolated mitochondria, different animals. Comparisons were made using ANOVA followed by Fisher 's test.

Materials

RR was obtained from Sigma. The actual concentration of the dye was calculated after determining the absorbance at 533 nm. No important spectrophotometric or toxicity differences were observed between different lots. Poly-L-lysine, MTT, procaine, caffeine, EGTA, ionomycin, $LaCl_3$, cycloheximide and actinomycin D, were purchased from Sigma. Dantrolene was from Norwich Eaton Pharmaceuticals (Norwich, NY). (+)-5-Methyl-10,11-dihydro-5H-dibenzo-(a,d)

cyclohepten-5,10-imine maleate (MK-801) and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX) were obtained from Research Biochemicals International (Natick, MA), and Fura 2-AM from Molecular Probes (Eugene, OR). The acetoxymethyl ester of *bis*-(o-aminophenoxy)-ethane-N,N,N',N'-tetracetic acid (BAPTA-AM, Calbiochem, La Jolla, CA) was dissolved in DMSO and added at a final concentration of 0.15% solvent. In this set of experiments, control cultures included 0.15% DMSO. Apoptosis detection kit was purchased from Oncor (Gaithersburg, MD).

<div align="center">

RESULTS

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Electron microscopy

As previously reported (Velasco et al., 1995; Velasco and Tapia, 1997), RR induced a clear dose-dependent neurodegeneration in cultured cortical neurons after 24 h of exposure. This damage was previously determined with biochemical techniques and light microscopy, and here we show the ultrastructural details of this neurodegeneration, with the aid of both scanning and transmission electron microscopy. As shown in Figs. 1 and 2, the most conspicuous alterations produced by RR occur in neurites, which lose their complex network and become distorted, thinner and with varicosities. These changes are already clear with 20 µM RR but are much more noticeable with 50 and 100 µM RR, until with the latter dose the neurite network has practically disappeared (Fig. 1). Notable changes occur also in neuronal somata, which show distinct degrees of damage evidenced by the scanning electron microscope (Figs. 1 and 2). The most prominent alteration is a disruption of

the cell membrane, which seems to follow several steps: a) roughening of the normally smooth surface (compare neurons of Fig. 1A, 2A and those marked "0" in Fig. 2B and 2C with those marked "1"); b) perforation of the membrane, as shown in the neurons marked with a curved arrow in Fig. 1B, C and D, and in those marked "2" in Fig. 2B and C; c) complete disruption of the membrane (neuron marked "3" in Fig. 2C) which leads to cell disintegration (thick arrow in Fig. 2C).

Further details of the ultrastructural damage of the neurons produced by 100 µM RR at 16 and 24 h can be seen in the transmission electron micrographs of Fig. 3. Some neurons are practically disintegrated, and in these cells, the nucleus looks condensed and more electron-dense, suggesting RR binding, especially in the nuclear membrane (arrows). However, this nuclear alteration is observed only when plasma membrane integrity is lost. No significant association of RR with the plasma membrane nor endocytotic images were observed. Interestingly, and in agreement with previous findings (Velasco et al., 1995; Velasco and Tapia, 1997), some cells were resistant to the damaging effect of RR, as can be seen in Figs. 1-3.

$[Ca^{2+}]_i$ changes

As mentioned in the Introduction, RR may exert some actions on $[Ca^{2+}]_i$ and therefore we tested if alterations in the homeostasis of this cation play a role in RR neurotoxicity, as assessed by MTT reduction. For this purpose, we used BAPTA-AM (a cell permeable form of a Ca^{2+} chelator), dantrolene and procaine (compounds that prevent intracellular Ca^{2+}-induced Ca^{2+} release) and caffeine (an alkaloid that releases Ca^{2+} from internal pools). Table 1 shows that none of these drugs altered

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significantly the RR-induced degeneration. Preincubation for 2 h with BAPTA-AM or dantrolene was also without effect. Similar negative results were found with cerebellar granule neurons (data not shown).

The possible participation of intracellular Ca^{2+} dishomeostasis in RR toxicity was also studied in a more direct way, by measuring the changes in $[Ca^{2+}]i$ (Fig. 4). The resting $[Ca^{2+}]i$ of control cells was always below 100 nM and the high potassium medium elicited an increase to about 400-500 nM. Neurons treated with RR showed higher resting $[Ca^{2+}]i$, reaching maximal values of about 300 nM after 2 h exposure to the dye. Furthermore, in the RR-treated neurons the high K^+-depolarization induced a $[Ca^{2+}]i$ increase higher than in control neurons in the first hour, reaching 500-800 nM concentrations. It is worth mentioning that RR-containing cells (as judged from their reddish appearance) had consistently higher $[Ca^{2+}]i$ and showed more susceptibility to degeneration, as evidenced by the complete loss of fluorescence at 100-180 min. The proportion of RR-containing neurons increased with exposure time.

Participation of Ca^{2+} channels in the induction of neuronal death

To discern if RR exerts its damaging effect through its well known Ca^{2+} channel-blocking action, we tested the effect of La^{3+}, another Ca^{2+} channel blocker that is able to displace RR binding to plasma membrane in synaptosomes (Tapia et al., 1985) on MTT reduction. Exposure of the cultures to 100 µM La^{3+} for 24 h caused a small, non-significant decrease in MTT reduction and slightly potentiated the neurotoxicity of 20 and 50 µM RR (Fig. 5). In some experiments, La^{3+} was tested at

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200 μM concentration. At this high dose, La^{3+} by itself induced neuronal damage (47.2 ± 5.3% decline in MTT reduction after 24 h, n=3), and morphologically (light microscopy) the neurons looked damaged but very different from those treated with RR: the cells showed vacuolization and no obvious alteration of neurites was observed (not shown).

RR effect on isolated mitochondria

Since RR can penetrate the neuronal plasma membrane at a time when no obvious damage is yet seen, it is possible that it may interfere with mitochondrial function. When we tested the effect of 100 μM RR on MTT reduction in isolated brain mitochondria, we found that the dye significantly inhibited this function (Fig. 6).

Effect of ionotropic glutamatergic receptor antagonists

It has been postulated that RR might act as an excitotoxin (Tapia et al., 1976; García-Ugalde and Tapia, 1991) because it induces seizures and other signs of hyperexcitation after its intracerebral administration, which are partially prevented by NMDA receptor antagonists (Belmar et al., 1995). Therefore, we attempted to prevent the RR-induced neurodegeneration in cultures with a combination of NMDA and non-NMDA glutamate receptor antagonists, MK-801 and NBQX. As shown in Table 2, this treatment had no effect on RR toxicity.

RR and apoptosis

Some morphological features observed in RR neurotoxicity, such as the nuclear condensation evidenced in Fig. 3, plus the prolonged exposure times

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required to observe neuronal damage, suggested that RR may induce apoptosis. However, the two experimental approaches to study this possibility did not support this: *in situ* labelling of DNA nicks did not reveal apoptotic cells in the RR-treated cultures (data not shown), and protein synthesis inhibitors did not prevent the RR-induced neuronal death (Table 3).

DISCUSSION

Scanning electron microscopy permitted to distinguish some characteristics of RR-induced damage in the plasma membrane. Since there are several states of destruction, as exemplified in Fig. 2, it is probable that RR causes progressive alterations that culminate in the rupture of the membrane. However, it is difficult to determine if the membrane damage is a primary event or it is a consequence of an intracellular action of the dye (see below). RR binds rapidly to plasma membrane in synaptosomes, mainly to Ca^{2+}-binding sites (Tapia et al., 1985; Tapia y Velasco, 1997). Further evidence of RR interaction with membranes comes from experiments with liposomes. It has been shown that RR can be adsorbed in multilamellar liposomes containing phosphatidylcholine and negatively charged phospholipids, such as phosphatidylserine, phosphatidylinositol or phosphatidylglycerol (Voelker and Smejtek, 1996). These authors calculated that when interacting with these charged liposomes, RR present a variable positive charge between 3+ to 5+, instead of the accepted 6+. Thus, RR could be interacting with phospholipids, monosialogangliosides (Velasco and Tapia, 1997), voltage-sensitive Ca^{2+} channels (Hamilton and Lundy, 1995; Tapia and Velasco, 1997) and/or unidentified proteins

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of the neuronal membranes.

In electron transmission micrographs no evidence of interaction of RR with plasma membrane of surviving cells was observed. In the damaged neurons, the cytoplasm appeared disintegrated and RR was clearly associated to the nucleus of damaged neurons, consistently with previous observations *in vivo* (Belmar et al., 1995). This is in agreement with our previous postulation that RR is internalized by susceptible cells (Velasco et al., 1995; Velasco and Tapia, 1997) and with the nearly linear correlation established between intracellular RR and neuronal damage (Velasco and Tapia, 1997). Furthermore, other effects of extracellularly applied RR imply a previous internalization of the dye. Among these, RR inhibits the mitochondrial $[Ca^{2+}]$ increases after NMDA application to striatal neurons in culture (Peng et al., 1998) and decreases the mitochondrial rhodamine 123 fluorescence after depolarization of sensory neurons with high potassium concentration (Duchen, 1992).

The present results on the measurement of $[Ca^{2+}]_i$ also support this hypothesis, since we observed that RR-treated cells showed elevated resting cytoplasmic Ca^{2+} and exacerbated $[Ca^{2+}]_i$ elevations in response to K^+-depolarization. These effects are likely to be exerted by intracellular RR, as shown in other neuronal types (Thayer and Miller, 1990; Marrion and Adams, 1992). However, the lack of protection or even potentiation by Ca^{2+} chelators or by blockers of the intracellular Ca^{2+} release observed here, suggest that Ca^{2+} dishomeostasis is not the only or main responsible for RR-induced neurodegeneration.

The present data with La^{3+} also suggest that RR is not acting primarily on plasma membrane Ca^{2+} channels to induce neurodegeneration. Since La^{3+} blocks RR binding (Tapia et al., 1985), one should expect that it should inhibit RR toxicity, but this cation slightly potentiated it. La^{3+} by itself caused neuronal death. It is therefore possible that La^{3+} action was due to a different, unknown mechanism. Finally, our negative results with glutamate receptor blockers indicate that ion fluxes through these types of channels are not involved in RR neurotoxicity.

The intracellular actions through which RR could induce neurodegeneration might be: 1) inhibition of mitochondrial function (Fig. 6; Vasington et al., 1972); 2) inhibition of Ca^{2+}-binding proteins such Ca^{2+}-ATPase (Vasington et al., 1972; Missiaen et al., 1990) or calmodulin (Sasaki et al., 1992; Charuk et al., 1990), proteins that are involved in maintaining low Ca^{2+} levels inside the cell; 3) production of free radicals, since it has been shown that RR can act as a Fenton-type reagent in the presence of ascorbate or respiring mitochondria (Meinicke et al., 1996); 4) disassembly of the microtubules formed by tubulin (Deinum et al., 1985; Velasco et al., 1995). The most likely of these are alterations in Ca^{2+} homeostasis and in normal mitochondrial function.

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FIGURE LEGENDS

Fig. 1. Scanning electron micrographs of cultured cortical neurons after 24 h exposure to RR. A: control. B: RR 20 μM. C: RR 50 μM. D: RR 100 μM. Note the concentration-dependent damage to the somata (arrows) and the progressive disintegration of neurites. Some cells were not damaged by RR. Scale bar = 10 μm. Images representative of 3 experiments with similar results.

Fig. 2. High magnification scanning electron micrographs of 100 μM RR neurotoxicity. A: control. B: RR exposure for 8 h. C: RR exposure for 24 h. The progressive damage to the soma is indicated by the numbers in the figure (0 indicates no damage and 3 very severe damaged cells). The thick arrow marks a neuron that has lost part of its membrane. Scale bar = 5 μm. Images representative of 3 experiments with similar results.

Fig. 3. Transmission electron micrographs of 100 μM RR neurotoxicity. A: control. B: RR exposure for 16 h. C: RR exposure for 24 h. The damaged neurons have lost their normal cytoplasmic architecture and the nuclei look condensed and with electron-dense deposits. RR association to nuclear membrane is pointed out by the arrows. Similarly to Figs. 1 and 2, some cells were unaffected by RR. Scale bar = 2 μm. Images representative of 3-4 independent experiments with similar results.

Fig. 4. 100 μM RR induces higher $[Ca^{2+}]_i$ in cortical neurons. Note that in RR-treated cells, both basal and high potassium (HK)-stimulated $[Ca^{2+}]_i$ are higher than the control, even very shortly after adding RR. Mean values ± SEM for 3-4 experiments.

Fig. 5. 100 μM La^{3+} potentiates 20 and 50 μM RR-related neurotoxicity. Note that although not statistically different from control, La^{3+} by itself diminishes MTT reduction. * P<0.05 relative to control. § P<0.05 relative to RR alone. Mean values ± SEM for 5 experiments.

Fig. 6. RR effect on MTT reduction by isolated brain mitochondria. * P<0.05 relative to control. Mean values ± SEM for 6 experiments.

TABLE 1. *Lack of effect of Ca^{2+}-related drugs on RR neurotoxicity.*

[RR], µM:	0	20	50	100
Dantrolene 30 µM (n=5)				
RR alone	100 ± 10.4	78.9 ± 4.4 *	53.3 ± 3.7 *	26.0 ± 3.2 *
RR + dantrolene	91.7 ± 4.9	69.0 ± 3.5 *	46.8 ± 3.4 *	28.3 ± 3.0 *
Procaine 2 mM (n=4)				
RR alone	100 ± 10.5	68.0 ± 4.8 *	58.9 ± 5.0 *	32.4 ± 3.7 *
RR + procaine	89.8 ± 6.8	74.5 ± 2.3 *	55.1 ± 2.0 *	33.4 ± 2.2 *
BAPTA-AM 50 µM (n=8)				
RR alone	100 ± 7.4	----	----	34.3 ± 1.9 *
BAPTA-AM	96.3 ± 6.0	----	----	37.5 ± 2.2 *
Caffeine 1 mM (n=4)				
RR alone	100 ± 5.4	68.7 ± 4.2 *	45.5 ± 5.1 *	31.6 ± 1.9 *
RR + caffeine	90.6 ± 7.1	58.7 ± 5.7 *	36.7 ± 2.7 *	31.7 ± 4.4 *

MTT reduction assays were done as described in experimental procedures after 24 h of incubation. Drugs were applied to cultures simultaneously with RR. The results are expressed a % of control. * $P<0.05$ relative to control (drug- and RR-free condition).

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**TABLE 2.** *Lack of protection by ionotropic glutamate receptor antagonists on RR neurotoxicity.*

| [RR], µM:	0	20	50	100
MK-801 10 µM + NBQX 100 µM (n=3)				
RR alone	100 ± 10.7	79.1 ± 2.9 *	63.0 ± 4.9 *	26.4 ± 2.7 *
RR + MK + NBQX	97.5 ± 2.0	76.4 ± 1.3 *	64.5 ± 1.3 *	27.9 ± 4.0 *

MTT reduction assays were done as described in experimental procedures after 24 h of incubation. Drugs were applied to cultures simultaneously with RR. The results are expressed a % of control. * $P < 0.05$ relative to control (antagonists- and RR-free condition).

TABLE 3. *Protein synthesis inhibitors do not modify RR neurotoxicity.*

[RR], µM:	0	20	50	100
Actinomycin D 400 ng/ml or Cycloheximide 2 µg/ml (n=4)				
RR alone	100 ± 3.7	77.8 ± 5.6 *	61.1 ± 1.8 *	27.8 ± 3.3 *
RR + actinomycin D	97.7 ± 5.0	77.5 ± 3.6 *	62.4 ± 5.4 *	24.7 ± 2.2 *
RR + cycloheximide	103.8 ± 6.8	86.8 ± 7.2 *	61.8 ± 8.6 *	22.5 ± 2.1 *

MTT reduction assays were done as described in experimental procedures after 24 h of incubation. Drugs were applied to cultures simultaneously with RR. The results are expressed a % of control. * $P < 0.05$ relative to control (drug- and RR-free condition).
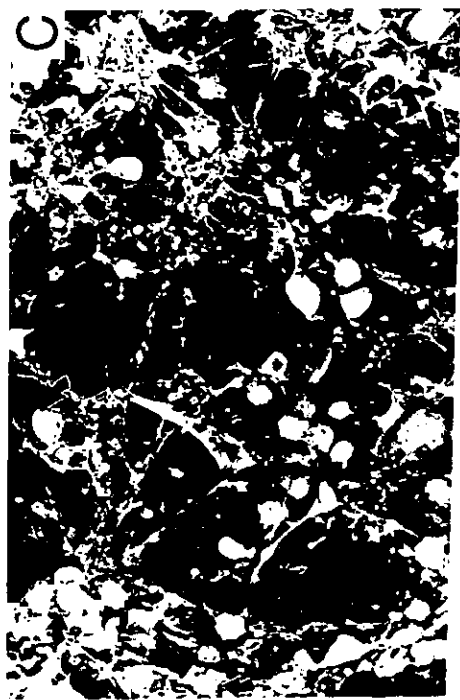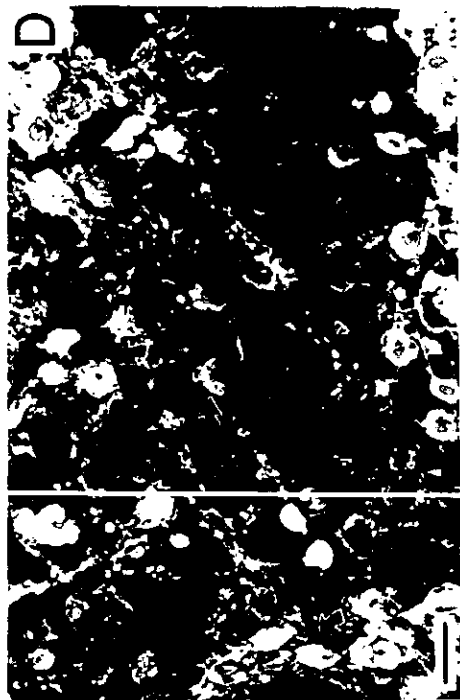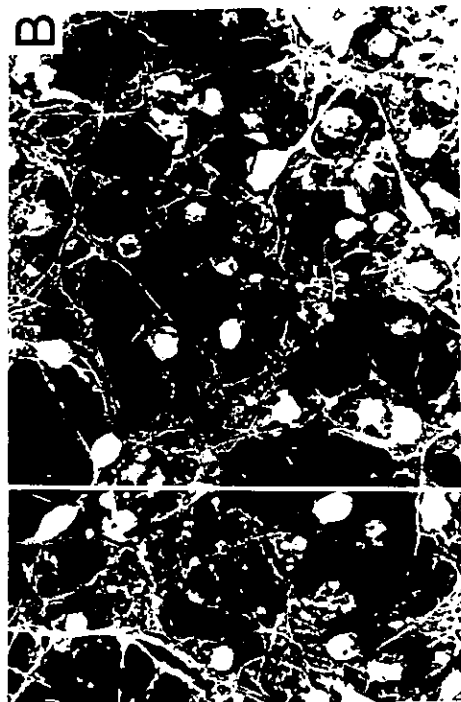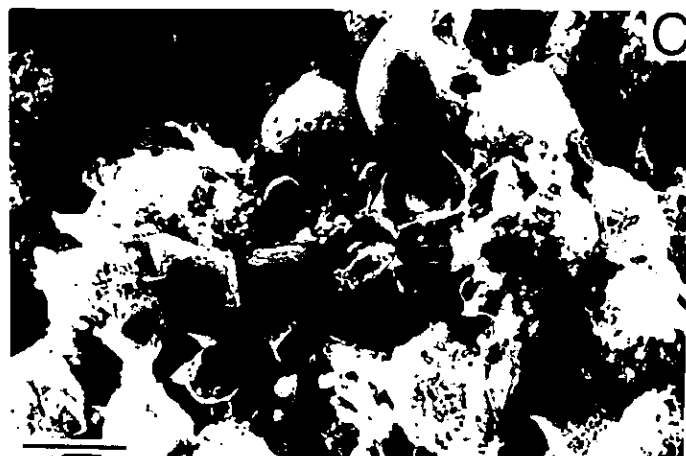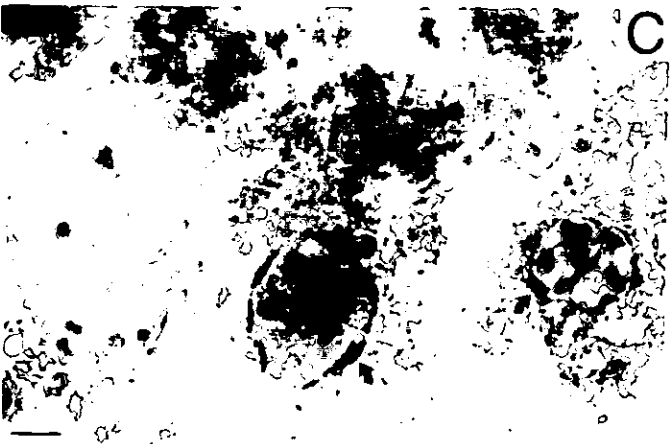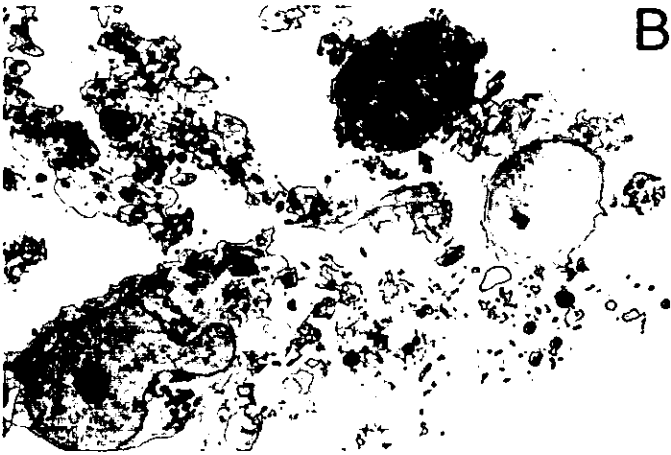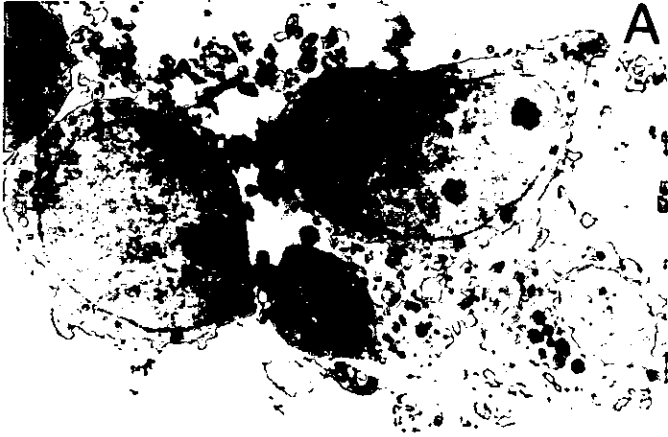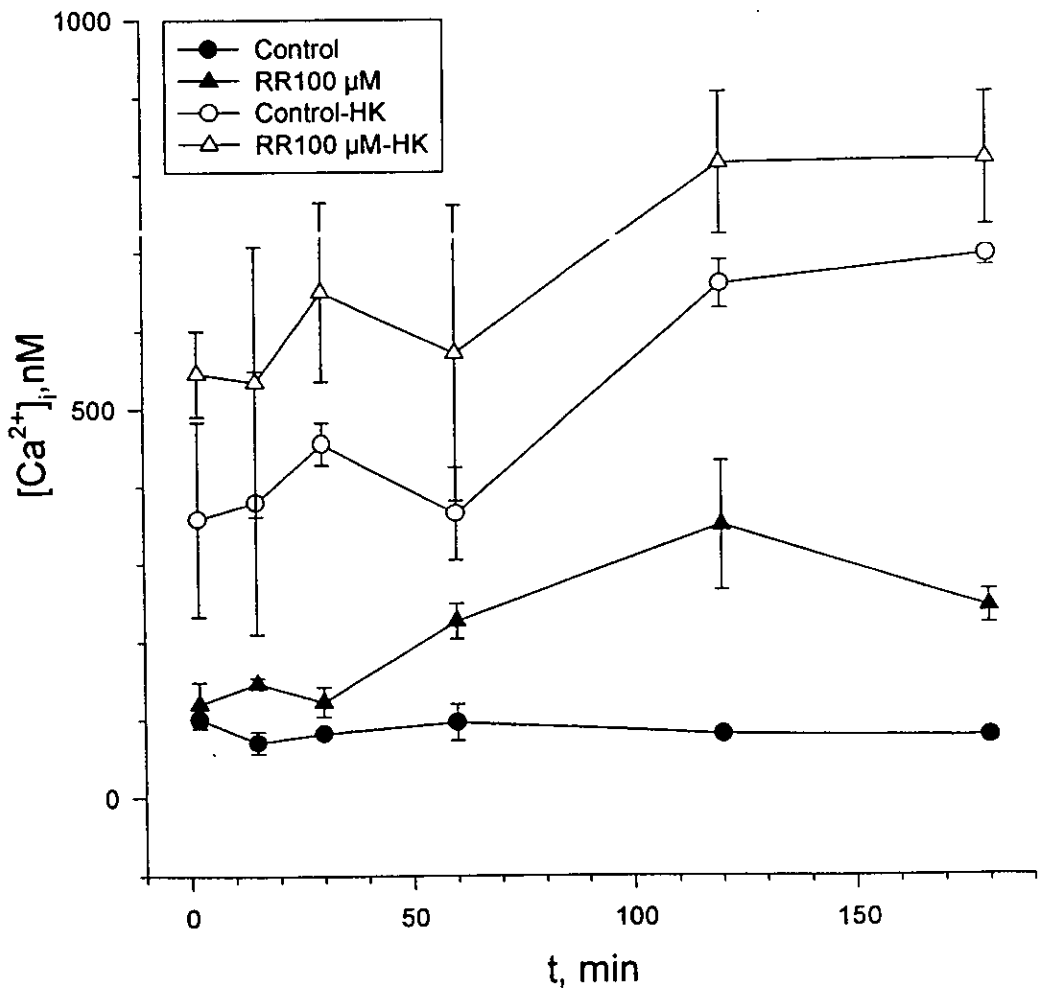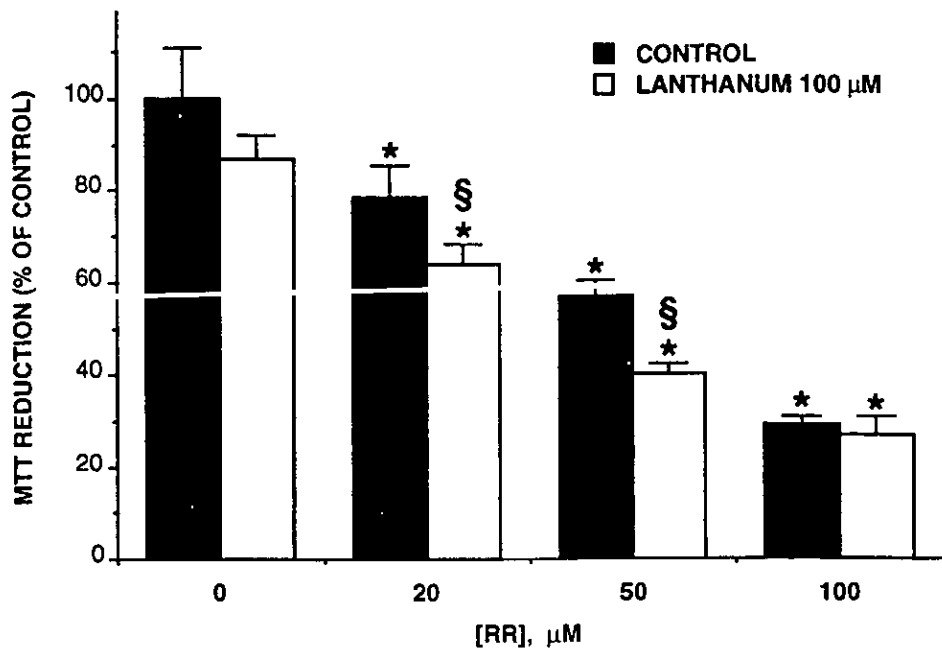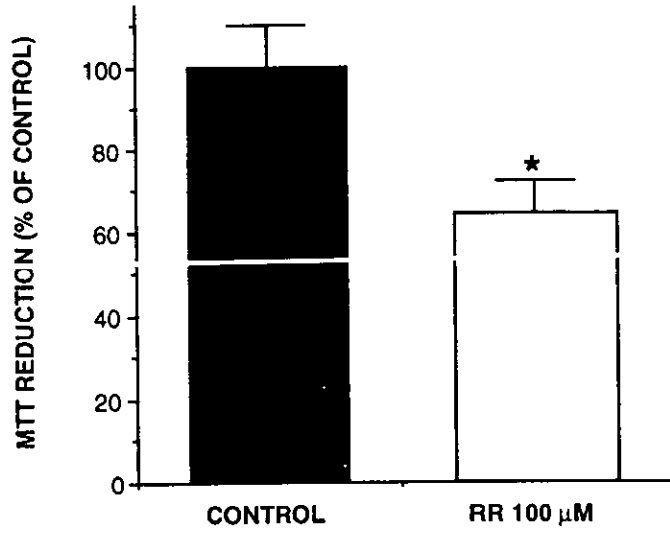
Injection of *Xenopus* Oocytes with mRNA from Cultured Neurons Induces New Currents and Susceptibility to the Damaging Action of Ruthenium Red*

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Running title: Damage by ruthenium red in neuronal mRNA-expressing oocytes.

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SUMMARY

The hexacationic dye ruthenium red (RR) produce neuronal death in primary cultures. We injected messenger RNA (mRNA) from cultured neurons into *Xenopus laevis* oocytes to test whether this treatment can make oocytes sensitive to the damaging action of RR. Two-microelectrode voltage clamp and resting membrane potential were used to evaluate mRNA expression and to assess the effect of RR on oocyte survival. The dye was added to the medium or injected into the cells, at 20, 50 or 100 µM concentrations. Injection of *Xenopus* oocytes with mRNA extracted from cultured cortical or cerebellar granule neurons produced 4 days later both new outward currents and membrane hyperpolarization. Exposure of mRNA-injected oocytes to extracellular RR for 24 h induced a remarkable depolarization, but no significant damage was observed. Injection of RR into buffer-injected oocytes did not cause any change in membrane potential or cell survival, whereas in mRNA-injected oocytes an important depolarization was observed 24 h after RR introduction, and 18-32% of the cells showed serious damage. The results suggest that oocytes become sensitive to intracellular RR toxicity because they express neuronal-specific proteins involved in cell death.

INTRODUCTION

Ruthenium red (RR[1]) is a well known Ca^{2+} transport blocker in isolated hepatic mitochondria (1,2). RR also inhibits the Ca^{2+} fluxes through the ryanodine receptor (3,4), and is a blocker of the voltage-sensitive Ca^{2+} channels, particularly the N and P types, in peripheral and central nerve endings (5,6). This dye can induce neuronal death both in vivo (7,8) and in primary cultures (9,10). In both cases it has been observed that the penetration of RR into neuronal somata seems to be a previous necessary event for the damage, which does not occur in glial cells. These results suggest that some properties of neuronal plasma membrane, not shared by glial cells, determine the entrance of RR into somata.

Xenopus laevis oocytes have been frequently used to express heterologous proteins after being injected with nucleic acids (11). Thus, messenger RNA (mRNA) extracted from central nervous system leads to the expression of functional receptors in the oocyte membrane, like the serotonin 2c (12) and the glutamatergic N-methyl-D-aspartate receptors (13). In the present study, we have injected *Xenopus* oocytes with mRNA extracted from cultured neurons, in order to assess whether this treatment could make oocytes sensitive to the toxic action of RR.

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[1] The abbreviations used are: RR, ruthenium red; mRNA, messenger RNA; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; I_{max}, maximal current.

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EXPERIMENTAL PROCEDURES

Neuronal culture and mRNA extraction

Neuronal cultures were prepared as described (9,10) using Wistar rats. Briefly, the cortices of 17 days-old fetuses and the cerebella of 8 days-old pups were digested with trypsin and seeded on plastic flasks (Costar, Cambridge, MA) in basal medium Eagle's (Sigma, St. Louis, MO) supplemented with 2 mM glutamine, penicillin (50 U/ml)/streptomycin (50 μg/ml) and 10% fetal bovine serum (Gibco, Gaithersburg, MD). Cerebellar granule neurons were grown in 25 mM KCl to promote their survival. Cytosine arabinoside was added 24 h after seeding to stop glial proliferation. The neurons were maintained in a 19:1 air:CO_2 incubator at 37°C for 4-6 days. After washing the culture medium with phosphate-buffered saline, the mRNA was extracted from 6×10^7 cells using a fast track 2.0 kit (Invitrogen, San Diego, CA), which employs oligo-dT cellulose. We performed mRNA isolation from two different cultures of cerebellar granule neurons and from one culture of cortical neurons. mRNA concentration was determined spectrophotometrically and adjusted to 1 μg/μl in the RNAase-free buffer included in the kit.

Oocytes extraction and injection

Oocytes were extracted as described (14,15) from adult *Xenopus laevis* anesthetized with tricaine. Clumps of cells were digested with collagenase for 2 h at room temperature in a medium containing 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), pH 7.4. Selected defolliculated oocytes were injected with 50 nl of the mRNA solution or with buffer

alone, transferred to ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 5 mM HEPES, 1.8 mM CaCl$_2$, 2.5 mM sodium pyruvate, gentamicin 100 µg/ml, pH 7.4) and maintained at 19°C during four days. We did not observe differences between buffer-injected and intact oocytes throughout the experiments described below.

Evaluation of mRNA expression

Changes in endogenous membrane currents and membrane potential were used as criteria of mRNA expression in oocytes. Four days after mRNA or buffer injection, whole cell currents were measured in the two-microelectrode voltage clamp mode as previously reported (14,15). Oocytes were placed at 23°C in a temperature-controlled chamber and step depolarizations (-80 mV to +50 mV in 10 mV increments) were applied from a holding potential of -100 mV. Electrodes were filled with 3 M KCl and had resistances below 1 MΩ. The data were digitalized by an analog/digital interface and recorded in a computer with the aid of pClamp 6 software (Axon Instruments, Foster City, CA). The leak current was subtracted off-line assuming ohmic leak.

Exposure to RR and evaluation of cell damage

Four to six days after the injection of either mRNA or buffer, oocytes were exposed to RR (Sigma, St. Louis, MO) in two different protocols: either adding the dye to the ND96 medium (20, 50 and 100 µM concentrations), or by injecting it into the oocytes dissolved in 50 nl of sterile water in concentrations calculated to give approximately 20, 50 and 100 µM RR inside the cells. For these calculations, oocytes were considered as perfect spheres of 1 mm diameter. Since commercial RR is not pure,

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actual dye concentrations were determined spectrophotometrically at 533 nm as described (8-10). After 24 h exposure to extracellular or intracellular RR, oocyte viability was evaluated by measuring the membrane potential. Oocytes with a membrane potential more positive than -10 mV were considered as significantly damaged. Several independent experiments using oocytes from different batches were carried out, and each experiment produced data averaged from at least 3 oocytes (normally 4-7 oocytes/condition). Statistical comparisons were made with ANOVA followed by Fisher's test.

RESULTS

Buffer-injected oocytes showed the previously described currents present in intact oocytes, where a Ca^{2+}-activated chloride conductance (16) is observed with depolarizations of the membrane to values between -10 mV to +30 mV (Fig. 1A). Four days after the oocytes were injected with mRNA from cortical neurons, and more clearly with mRNA from cerebellar granule cells, novel outward currents were apparent, which were not present in the control buffer-injected oocytes. In the case of oocytes injected with mRNA from cortical neurons these new currents did not inactivate during the 900 ms-depolarizing pulse, whereas in those injected with cerebellar mRNA, two components of the currents were observed, a fast one which inactivated in approximately 50 ms, and a slow one that did not inactivate importantly (Fig. 1B and 1C). The maximal elicited current (I_{max}) at +50 mV was significantly greater in oocytes injected with mRNA than that present in buffer-injected cells (Table I).

Exposure of oocytes to extracellular RR caused the appearance of large dark spots surrounded by white halos in the animal pole, and smaller reddish spots in the vegetal pole, visible in the stereoscopic microscope. These deposits, presumably accumulations of the dye, were apparently located between the plasma and the vitelline membranes. This pattern was observed in all cells exposed to extracellular RR, including the intact, buffer-injected and mRNA-injected oocytes.

As shown in Fig. 2, introduction of neuronal mRNA into oocytes induced a notable membrane hyperpolarization that did not occur in buffer-injected cells. The

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effect is clearer, as in the case of newly expressed currents, with mRNA from cerebellar neurons (-87 mV) than with mRNA from cortical cells (-56 mV). Exposure of these injected oocytes to RR in the medium (extracellular RR) caused a dose-dependent depolarization after 24 h, whereas the buffer-injected cells showed only a slight, non significant depolarization. The maximal membrane potential changes were 12 mV in buffer-injected cells, 33 mV in cells with cortical neurons mRNA and 61 mV in oocytes with cerebellar mRNA (Fig. 2).

The intracellular injection of RR did not cause any apparent staining of the oocytes in any case. In the buffer-injected oocytes intracellular RR did not affect the membrane potential. In contrast, in the cerebellar mRNA-injected oocytes, RR induced an important depolarization, even at 20 μM RR, the lowest concentration tested. This effect seems to be maximal, since higher RR doses were equally effective (Fig. 3). In one experiment in which 11 oocytes were injected with mRNA from cortical neurons, intracellular RR produced changes in membrane potential similar to those shown in Fig. 3 (not shown).

Using the criterion indicated in Methods that a membrane potential $\geq$ -10 mV was indicative of significant damage, exposure to 20-100 μM extracellular RR did not induce any damage, either in the buffer-injected oocytes or in the cerebellar or cortical neuronal mRNA-injected oocytes (number of damaged cells/number of cells tested: buffer, 0/54; cortical mRNA, 1/31; cerebellar mRNA, 3/69). In contrast, when RR was injected into the cells, 18-32% of neuronal-protein expressing oocytes were damaged (buffer, 0/39; cortical mRNA, 2/11; cerebellar mRNA, 14/44).

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DISCUSSION

The novel findings of this work are the following: 1) injection of mRNA from cultured neurons into oocytes can lead to the expression of new currents and to a more hyperpolarized membrane potential; 2) exposure of buffer-injected oocytes to RR, either in the medium or in the cytoplasm, has minimal effects on membrane potential and cell survival; 3) in contrast, oocytes expressing neuronal mRNA were susceptible to RR toxicity, especially after introduction of the dye into the cells.

The new outward currents expressed by the mRNA-injected oocytes, particularly those induced by mRNA from cultured cerebellar granule neurons, resemble the K+ conductances described in oocytes injected with mRNA from whole adult brain (17), as well as the K+ fluxes observed in cultured cerebellar granule neurons grown in high (25 or 40 mM) extracellular K+ (18). Although we did not perform experiments to test if the novel currents were effectively due to K+ channels, the similarity of the changes in membrane properties strongly suggest that this is the case, and that these currents are due to the expression of neuronal mRNA in the oocytes. These new currents might be responsible for the observed membrane hyperpolarization.

The notable and dose-dependent depolarization from the initial hyperpolarized value induced by RR in neuronal mRNA-injected oocytes suggests that the expression of neuronal proteins by the oocytes renders these cells more susceptible to the action of RR. This conclusion gains further support from the results of the intracellular injection of RR on oocyte survival. In these experiments we did not observe any effect on the membrane potential in buffer-injected oocytes. In sharp

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contrast, the cells injected with neuronal mRNA showed a more depolarized membrane potential and about one third of the cells treated reached a very positive membrane potential (> -10 mV).

The findings of the present work suggest that extracellular action of RR on membrane potential is greatly potentiated by newly expressed neuronal proteins, whereas, if the dye is inside the cell, such expression is linked to cell damage. We have previously shown that RR can enter living neurons in culture (9) and that the extent of damage is directly related to RR uptake (10). Therefore, and since extracellular RR did not induce damage, the present results indicate that neuronal proteins, both in neurons and in oocytes, do not mediate the internalization of the dye but are involved in the RR-induced damage.

Future experiments might lead to the identification of the protein(s) possibly involved in making the neuronal expressing-oocytes susceptible to the damaging actions of RR. The procedure described here has the advantage of permitting the extraction of large amounts of poly-A RNA from a homogeneous population of cultured neurons and could be used to study the participation of unidentified proteins in several processes.

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FIGURE LEGENDS

Fig. 1. Ionic currents elicited by successive depolarizations (lasting 900 msec) of oocytes membrane potential, four days after injection of buffer or mRNA. A) Buffer-injected oocyte, where the endogenous Ca^{2+}-activated chloride conductance is apparent (-10 mV to +30 mV). B) Oocyte injected with mRNA extracted from cortical neurons. In addition to the mentioned chloride current, a new, sustained current is present with depolarizations above 0 mV. C) Oocyte injected with mRNA of cerebellar granule neurons, where two components of the novel ionic conductances can be distinguished. Note that the calibration bars for the current are different. The bottom record indicates the membrane potential. The presence of new currents in neuronal mRNA-injected oocytes were confirmed in 5-7 independent experiments.

Fig. 2. Effect of extracellular RR on membrane potential of oocytes injected with buffer or with mRNA from either cortical or cerebellar granule neurons, evaluated after 24 h exposure of the cells to the dye in the medium. Mean values ± SEM of 3-4 experiments (3-7 oocytes per experimental condition). * $P < 0.05$ relative to its own control (RR-free medium). § $P < 0.05$ relative to buffer-injected control.

Fig. 3. Effect of intracellular RR on membrane potential of oocytes injected with buffer or with mRNA from cerebellar granule neurons, evaluated 24 h after injection of the dye. Mean values ± SEM of 3 experiments (3-7 oocytes per experimental condition). * $P < 0.05$ relative to its own control (RR-free medium). § $P < 0.05$ relative to buffer-injected control.

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TABLE I

Ionic currents induced by membrane depolarization to +50 mV in buffer-injected and mRNA-injected oocytes

Injection	I_{max} at +50 mV (μA)
Buffer	0.08 ± 0.01
mRNA from cortical neurons	0.60 ± 0.07[a]
mRNA from cerebellar granule neurons	6.68 ± 0.71[a]

[a] $p < 0.05$ relative to control.

Oocytes were injected with buffer or mRNA from either cortical or cerebellar granule neurons. After four days, two microelectrode voltage clamp was used to depolarize the oocytes membrane to +50 mV from a holding potential of -100 mV. Values are means $\pm$ SEM from 5-7 independent experiments.

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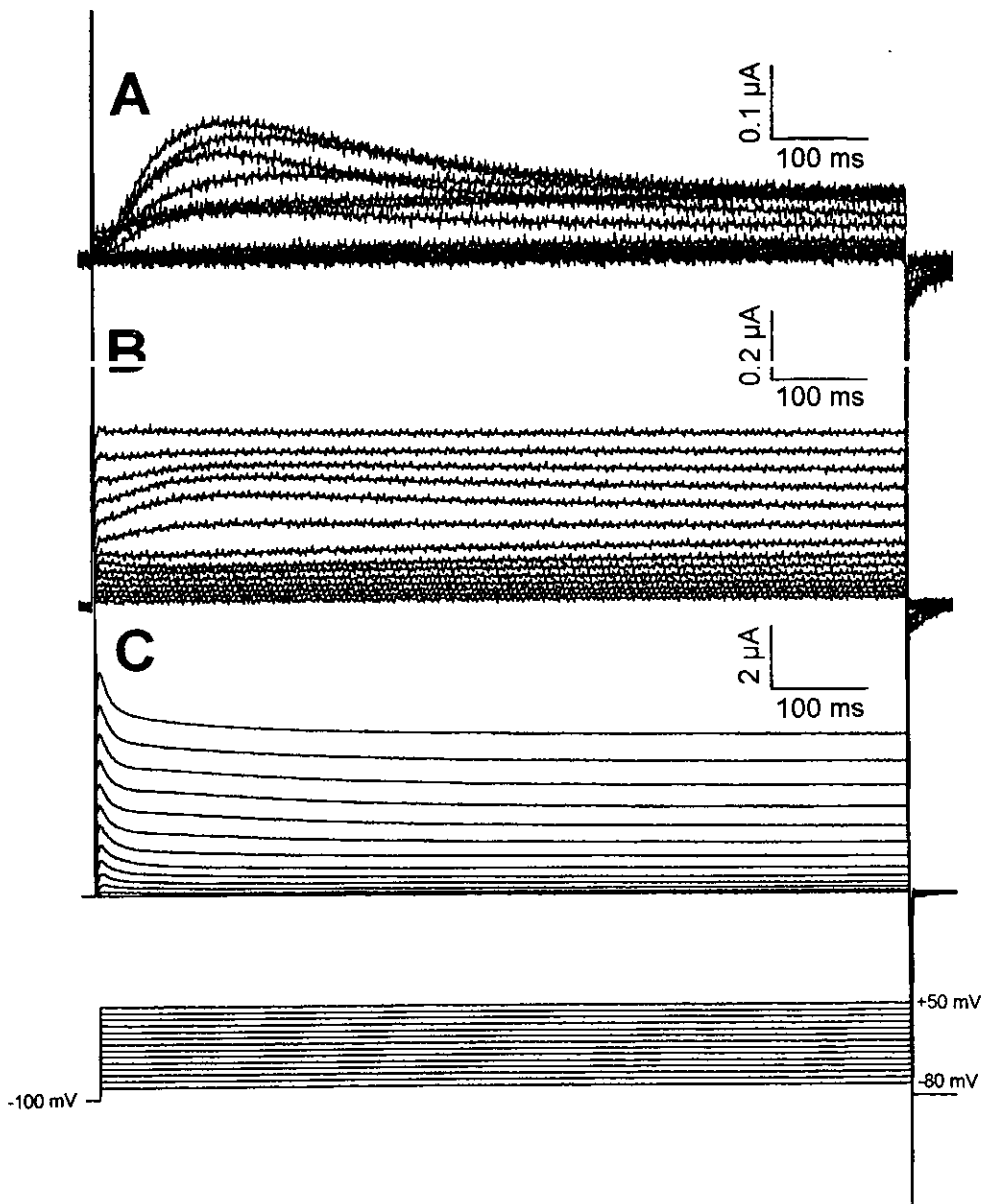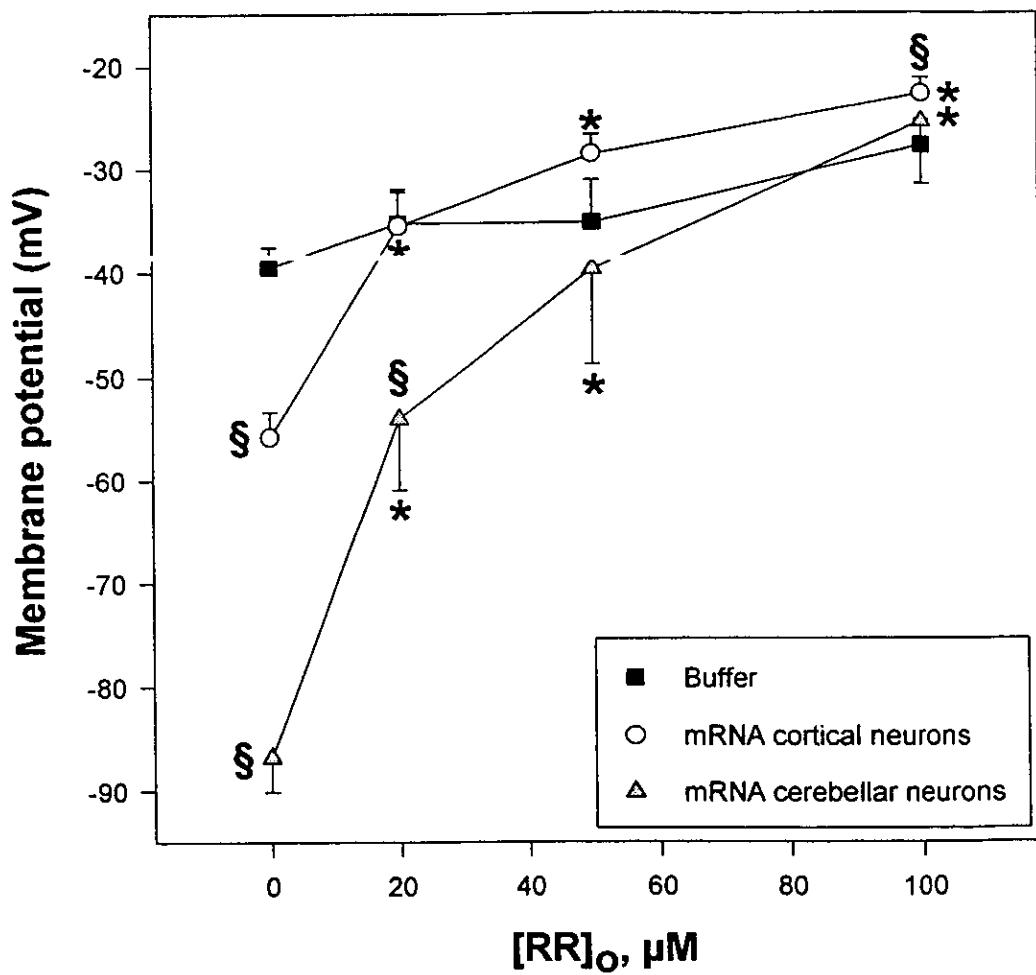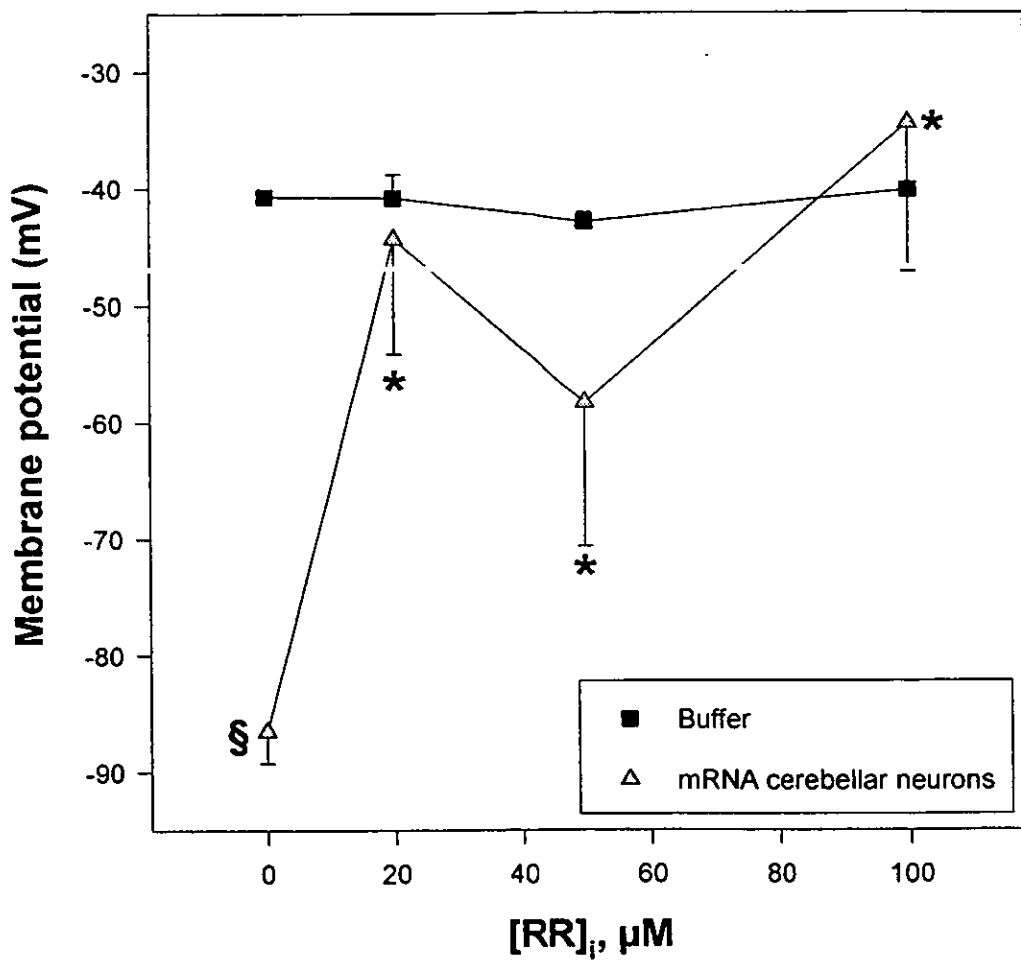
DISCUSIÓN GENERAL

Puesto que en los artículos que conforman esta tesis se discuten ampliamente los resultados obtenidos, el objetivo de esta discusión general es tratar de integrar brevemente todos los hallazgos y llegar a conclusiones puntuales.

En los trabajos incluidos en esta tesis se trató de establecer las alteraciones que produce el RR sobre las neuronas en cultivo. El primer fenómeno, que aparece pocas horas después del tratamiento con RR, es la penetración del colorante a los somas neuronales. Luego ocurren alteraciones morfológicas evidentes, como el adelgazamiento y la desaparición de las neuritas y la pérdida de la estructura membranal normal, observadas con microscopía óptica y electrónica de barrido. Este daño se correlaciona con una pérdida importante de la capacidad de las células para reducir el MTT por la cadena respiratoria, luego de 8-24 horas de incubación. La susceptibilidad neuronal al RR no depende del tipo de neuronas (corticales o granulares de cerebelo se afectan de manera muy similar) o de la edad del cultivo (artículos 2, 3 y 4).

En contraste con las observaciones *in vivo*, la neurotoxicidad inducida por RR en los cultivos primarios requirió un mayor tiempo para manifestarse claramente: mientras que en la administración intrahipocámpica hay daño evidente a los pocos minutos después de la inyección del colorante, en los cultivos fue necesario hacer incubaciones de varias horas poder observar dicho fenómeno. Esta diferencia puede deberse a que las características de las neuronas de adulto y las fetales o postnatales en cultivo no sean las mismas. Sin embargo, las alteraciones

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ultraestructurales causadas por el RR, observadas usando microscopía electrónica de transmisión, son muy similares *in vivo* y en los cultivos corticales (ver Belmar et al., 1995 y artículo 4): en ambos casos se observó daño citoplasmático y asociación del RR con el núcleo de las neuronas dañadas. Otra similitud entre las observaciones *in vivo* e *in vitro* la consituye la penetración del RR a los somas neuronales (Tapia y Flores-Hernández, 1990; García-Ugalde y Tapia, 1991; Belmar et al., 1995; artículo 2).

La internalización del RR a las neuronas en cultivo parece ser un paso determinante en la neurotoxicidad por RR, debido a que en los cultivos de glía no se observó penetración del colorante ni daño celular (artículo 2). Sin embargo, la internalización del colorante no induce por sí misma daño celular, puesto que ovocitos control de *Xenopus laevis* a los que se les inyectó RR no mostraron signo de deterioro alguno. En contraste, cuando los ovocitos expresaban mRNA neuronal, mostraron una mayor susceptibilidad a la acción tóxica del RR (artículo 5).

Desafortunadamente, no pudimos identificar el mecanismo mediante el cual el RR se introduce a las neuronas. Aunque una posibilidad obvia es la endocitosis, las observaciones ultraestructurales nunca mostraron imágenes sugerentes de este mecanismo (artículo 4). En los primeros trabajos con RR, realizado en amibas (Szubinska y Luft, 1971), se encontró que el RR ocasiona la muerte celular en 2-30 minutos, con concentraciones de 0.6 a 1.2 mM. Los cambios que se presentaron luego de la exposición al colorante fueron: retracción de pseudopódos, mayor fragilidad al contacto y después de 15-20 minutos, movimiento browniano en el citoplasma. El RR no pudo ser despegado de la membrana plamática mediante lavados y aunque

ésta no sufrió cambios notables, el colorante fue capaz de penetrarla, puesto que se encontró RR dentro de las células; la presencia de estos gránulos de colorante fue atribuida a pinocitosis.

En el caso de las neuronas en cultivo, se ha observado que hay exocitosis en la liberación de neurotransmisor y endocitosis de vesículas por la célula presináptica (Ryan y cols., 1993), por lo que es posible que el RR se introduzca a las células por este mecanismo. Sin embargo, esta posibilidad no se ve apoyada puesto que una exposición corta de neuronas cultivadas a RR 20 µM no causó alteraciones en la presinapsis (Trudeau y cols., 1996) y nosotros nunca observamos tinción evidente en las neuritas.

Una diferencia importante entre las neuronas y los astrocitos en cultivo es el hecho de que las primeras tienen una mucha mayor cantidad de canales iónicos (Dichter, 1978; Choi y cols., 1987) que las células gliales (Sontheimer, 1994), lo que abre la posibilidad de que el RR se internalice a las neuronas al pasar por un canal catiónico no identificado. Por otra parte, las membranas de las neuronas cultivadas contienen más gangliósidos (Ogiso y cols., 1992; Thangnipon y Balázs, 1992) y presentan una mayor proporción de gangliósidos con varios residuos de ácido siálico (Dreyfus y cols., 1980; Thangnipon y Balázs, 1992), comparadas con la membranas de los astrocitos en cultivo. Sin embargo, sabemos que la eliminación de los residuos de ácido siálico de sialoproteínas y polisialogangliósidos en la membrana plasmática con neuraminidasa no tiene efecto sobre la penetración del colorante a las células o el daño inducido por RR (artículo 3).

Otra posibilidad interesante para explicar la entrada del RR a las neuronas es que la composición de fosfolípidos de la membrana neuronal sea la responsable de la entrada selectiva del RR, puesto que se ha demostrado que el colorante puede ser adsorbido por liposomas que contienen fosfatidilcolina y alguno de los siguientes fosfolípidos con carga negativa: fosfatidilserina, fosfatidilglicerol o fosfatidilinositol (Voelker y Smejtek, 1996). En este trabajo también se encontró que la valencia efectiva del RR (que teóricamente es 6+), depende de la densidad de carga de los liposomas, y se encontraba entre 3+ y 5+, lo que podría hacer más fácil la entrada del colorante. No obstante que no es fácil imaginarse que una molécula tan grande y cargada pueda pasar la membrana celular, hay trabajos en los que al adicionar RR al medio se observan efectos sobre la mitocondria a tiempos cortos (minutos), lo cual sugiere que el colorante se introdujo a las neuronas (Duchen, 1992; Peng y cols., 1998).

En diversas preparaciones del SN el RR inhibe, a concentraciones micromolares, la liberación de neurotransmisores dependiente de Ca^{2+} (ver artículo 1). La participación del bloqueo por RR de los VSCC en la neurodegeneración de las células cultivadas parece ser mínima. Primero, el bloqueo de dichos canales por el RR tiene un efecto protector contra las elevaciones de $[Ca^{2+}]_i$ (Duchen, 1992) y sobre la neurotoxicidad, como se ha descrito en el caso de la excitotoxicidad por Glu o sus análogos en cultivo (Dessi y cols., 1995; Eimerl y Schramm, 1995). Segundo, al tratar de desplazar al RR, con La^{3+}, de los sitios a los que se une en la presinapsis, como se ha observado en sinaptosomas (Tapia y cols., 1985), no encontramos una

disminución sino una ligera potenciación de la toxicidad (artículo 4), a pesar de que era de esperarse un desplazamiento de aproximadamente 40% de la unión de RR 20 μM en presencia de La^{3+} 100 μM en los sitios que comparten en la terminal sináptica. Es interesante mencionar que al incubar a los cultivos corticales con La^{3+} 200 μM durante 24 horas, se observó muerte celular importante ($\approx$50%), lo que sugiere, junto con la potenciación de la toxicidad por RR, que el bloqueo de los VSCC puede producir muerte neuronal, o que el La^{3+} está actuando en sitios no identificados que conducen a la neurodegeneración. En tercer lugar, al prevenir la captura/unión del RR en los cultivos con el trisialogangliósido GT1b, la muerte neuronal fue evitada parcialmente, lo que no se observó con el monosialogangliósido GM1.

Por otro lado, Baux y cols. (1979) encontraron que el efecto inhibitorio del RR sobre la liberación de transmisores al inyectar colorante dentro de neuronas ganglionares de *Aplysia californica*, podía ser revertido al tratar a las células con neuraminidasa intracelular. Su conclusión fue que el RR, al unirse a los sitios que contienen ácido siálico y que unen Ca^{2+} en el interior de la neurona, impide la exocitosis del neurotransmisor. Además, Wierasko (1986) encontró que al superfundir rebanadas de hipocampo con RR, se impedía la transmisión sináptica, lo que podía retrasarse con la adición de una mezcla de gangliósidos (con 2 ó 5 veces más concentración de gangliósidos que de RR), ácido siálico (con 14 veces más siálico que colorante) o con el tratamiento de las rebanadas con neuraminidasa. Tomando en cuenta estas diferencias, y apoyado en otras observaciones antes descritas, se

puede concluir que la capacidad del RR para bloquear la salida de neurotransmisores no está relacionada de manera importante con la inducción de la muerte neuronal.

El RR (10 μM) en el medio puede disminuir la inactivación del canal de Na^+ presente en neuronas de caracol, además de bloquear los VSCC como en muchas otras preparaciones (Stimers y Byerly, 1982). Estos autores encontraron que el colorante disminuía la resistencia membranal y las corrientes salientes dependientes de voltaje. Recientemente se ha descrito que el RR puede disminuir la corriente de K^+ activada por Ca^{2+} (IC_{50}=4.2 μM) en músculo liso, probablemente al unirse al sitio de unión de Ca^{2+} del canal y disminuir de ese modo la probabilidad de apertura (Hirano y cols., 1998). La contribución de estos efectos del RR a la neurodegeneración observada en cultivos de rata se desconoce. No obstante, todas estas acciones del colorante podrían llevar a una pérdida de los equilibrios iónicos que se requieren para que una célula se mantenga viva. Es evidente que el dilucidar las acciones del RR sobre los canales iónicos de las neuronas cultivadas sólo se podría hacer mediante el registro electrofisiológico de las células, lo que no se pudo realizar en este trabajo.

Se ha propuesto que el RR podría actuar como una excitotoxina, puesto que luego de su inyección en el parénquima cerebral induce muerte neuronal y alteraciones conductuales asociadas con hiperexcitación (ver artículo 1). Además, recientemente se ha publicado que el RR puede inducir la liberación sináptica de Glu independiente de Ca^{2+} en neuronas hipocampales en cultivo (Trudeau y cols., 1996). Decidimos probar entonces si antagonistas de los receptores ionotrópicos a Glu

podían proteger a las neuronas corticales de la toxicidad por RR, y encontramos que la excitación debida a ese grupo de receptores no participa en la neurodegeneración por RR (artículo 4).

IV. i. Posibilidades para explicar el daño neuronal por RR

Los mecanismos de la neurotoxicidad del RR se esquematizan en la figura 3. A lo largo de los trabajos presentados, se apoya la hipótesis de que el RR debe entrar a las neuronas para producir daño celular. Varias son las posibilidades mediante las que el RR podría entrar a las neuronas:

• Endocitosis (1).

• Un canal catiónico (2)

• Difusión pasiva favorecida por el gradiente de concentración (3).

• Un transportador dependiente o independiente de energía (4).

• Adsorción a la membrana al interaccionar con las cargas negativas de la superficie neuronal (por ejemplo gangliósidos, 5).

De todas estas opciones, me parece que la más viable puede ser una difusión facilitada por el gradiente de concentración luego de que el RR ha interaccionado con componentes membranales con carga negativa. Cualquiera que sea el mecanismo de internalización, éste no está presente en las células gliales.

Entre las acciones dentro de la célula que podría ejercer el RR y que contribuirían a la muerte, se incluyen:

• El impedimento de una eficaz disminución de la $[Ca^{2+}]_i$ luego de un aumento en la concentración intracelular de este catión (6; Thayer y Miller, 1990; Marrion y Adams,

1992). A pesar de que en nuestro sistema la muerte neuronal no se evitó al usar compuestos que impiden la elevación de $[Ca^{2+}]_i$, encontramos que, luego del tratamiento con RR, las neuronas tenían una $[Ca^{2+}]_i$ basal más alta que los controles (artículo 4). Aún más, la despolarización de las células tratadas con RR ocasionó una elevación exacerbada de $[Ca^{2+}]_i$, no obstante que el medio contenía RR. Dos posibilidades pueden explicar este resultado: 1) el colorante, una vez dentro de la neurona, está interfiriendo con la homeostasis del Ca^{2+}, como se ha descrito (Thayer y Miller, 1990; Marrion y Adams, 1992); 2) el RR está promoviendo una mayor entrada de Ca^{2+} a las células (lo que estaría en contraposición con lo reportado en la literatura). Así, al parecer el RR se introduce a las células y es capaz de impedir un correcto amortiguamiento de la $[Ca^{2+}]_i$, aunque la elevación de los niveles basales de Ca^{2+} en las neuronas tratadas con RR podría ser reflejo de otras alteraciones. A este respecto, se ha demostrado que el RR es capaz de interferir con la función de las ATPasas de SR (Madeira y Antunes-Madeira, 1974), de la mitocondria (Vasington y cols., 1972) y de la membrana plasmática (Missiaen y cols., 1990). La acción del RR sobre estas proteínas podría tener como consecuencia la elevación de la $[Ca^{2+}]_i$.

• La desestabilización de las proteínas que conforman el citoesqueleto, especialmente la tubulina (7). Se ha demostrado que el RR puede impedir la formación de microtúbulos de esta proteína y promover su despolimerización, además de interferir con la función de transporte que tiene el citoesqueleto (Deinum y cols., 1981). En nuestros cultivos, observamos una pérdida muy importante de la inmunoreactividad para α-tubulina (artículo 2). Sin embargo, no pudimos establecer

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si este efecto es causa o consecuencia de la muerte neuronal. Además, las neuritas no se dañan tanto con RR como los somas (artículo 4), lo cual no apoya un efecto generalizado del colorante sobre componentes del citoesqueleto.

• La producción de radicales libres (8). El RR en presencia de mitocondrias funcionales es capaz de producir ROS (Meinicke y cols., 1996), las cuales se han involucrado en daño a los componentes celulares, que finalmente conducen a la muerte celular. Experimentos preliminares en este sentido indican que el RR no induce la producción de radicales libres en las neuronas cultivadas.

• El interferir con la función mitocondrial normal (9). En esta tesis se mostró que el RR puede disminuir el funcionamiento de la cadena de transporte de electrones de la mitocondria (artículo 4), lo cual es consistente con su capacidad de inhibir la respiración en mitocondrias hepáticas (Vasington y cols., 1972).

De las posibles acciones intracelulares del RR, las que me parecen más probables son la afección de la homeostasis de Ca^{2+}, la inhibición de la función mitocondrial y la inhibición de alguna proteína vital neurona-específica no identificada (10).
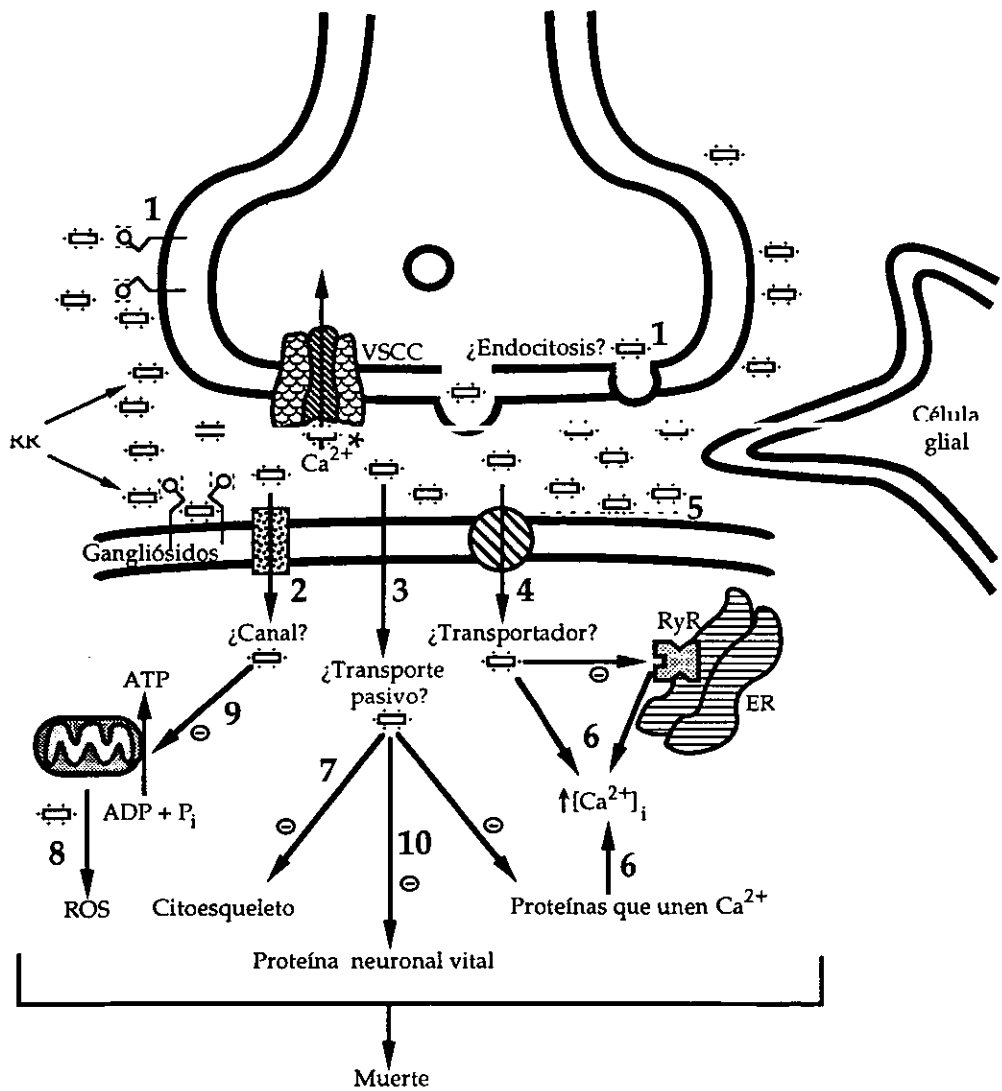
Figura 3. Mecanismos de la neurotoxicidad por RR en neuronas cultivadas. Los números indican los sitios probables de acción del RR para provocar la neurodegeneración. En el texto se discute la posible participación de cada uno y se establecen las hipótesis más viables. Con * se señala el bloqueo de los VSCC por RR, el cual no participa en la neurotoxicidad. Las abreviaturas se definen en la página iii.

IV. ii. Conclusiones

El RR causa muerte neuronal en cultivos primarios. Otros tipos celulares como astrocitos en cultivo u ovocitos no son susceptibles a la acción tóxica del colorante.

La penetración del colorante a las neuronas (observada después de pocas horas) parece ser un paso determinante para la inducción de la muerte. Neuronas que no internalizan al RR no son afectadas por este compuesto.

El trisialogangliósido GT1b disminuye el daño neuronal ocasionado por RR al impedir la interacción del colorante con la membrana.

Las alteraciones ultraestructurales más evidentes ocasionadas por el RR sobre las neuronas cultivadas luego de 8-24 horas son la desaparición de las neuritas, daño en los somas y asociación del colorante con el núcleo.

El RR induce pérdida de la función mitocondrial tanto en neuronas como en mitocondrias aisladas.

El RR provoca la pérdida de la homeostasis de Ca^{2+} intracelular en las neuronas cultivadas luego de pocas horas.

El bloqueo de los canales de Ca^{2+} por el RR no es la principal causa de la neurotoxicidad.

El RR induce muerte necrótica en las neuronas cultivadas.

El uso experimental del RR para estudiar la muerte neuronal es interesante puesto que es una toxina selectiva que permite estudiar los mecanismos involucrados en la neurodegeneración.

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